S P E C I F I C A T I O N

Title of the Invention

PROCESS FOR PRODUCING PEPTIDES BY USING *in vitro*

TRANSCRIPTION／TRANSLATION SYSTEM

Background of the Invention

1. Technical Field to which the Invention Belongs

[0001] This invention relates to a process for producing a

peptide or a peptide derivative by using a reaction system of

transcribing a DNA into an RNA and then translating the RNA

produced or a reaction system of translating an RNA *in vitro*

(hereinafter referred to as "in vitro transcription ／

translation reaction system") and a kit of protein components

which comprises enzymes and factors for the constitution of this

reaction system.

2. Description of the Related Art

[0002] There have been known cell-free protein synthesis

systems derived from *Escherichia coli*, rabbit reticulocytes,

or wheat germ (Current Opinion in Biotechnology 9:534-548

(1998), J. Biotechnology 41:81-90 (1995)). In these cell-free

systems, peptides can be synthesized within several hours.

1

Namely, proteins can be synthesized within a short time compared with the case where foreign genes are inserted into host cells and then expressed therein (Proc. Natl. Acad. Sci. USA 94:412-417 (1997), FEBS Letters 414:268-270 (1997)). Moreover, it is recognized or expected that synthesis of proteins in cell-free systems has a number of technical advantages, at least theoretically, over the case of inserting foreign gene into host cells and expressing the same. Namely, use of these cell-free protein synthesis systems makes it possible to produce peptides which would be digested by proteases originating in host cells and peptides showing toxicity on host cells. It is also possible by using these systems to produce peptide derivatives which do not occur in nature by incorporating unnatural amino acid residues into specific positions by using aminoacyl-tRNA charged by the unnatural amino acid residues (Annu. Rev. Biophys. Biomol. Struct. 24:435-462 (1995)), or complexes (polysome displays) composed of mRNA, ribosome and peptide. These polysome displays and utilization thereof are reported by He M.. et al., J. Immunological Methods 231 (2000) pp. 105-117, Schaffitzel C., J. Immunological Methods 231 (2000) pp. 119-135, Roberts RW., Current Opinion in Chemical Biology 3 (1999) pp. 268-273 and ibid. 9 (1998) pp. 534-548 (in particular, on and after p. 543) and, in addition, described in, e.g., FEBS Lett. 450:105-110 (1999), Proc. Natl. Acad. Sci. USA 95:14130-14135 (1998), and Proc. Natl. Acad. Sci. USA 94:4937-4942 (1997).

[0003] Although crude cell extracts *per se* were employed at the early stage, only unstable reactions could be performed thereby and thus peptides were synthesized only at low yield, namely, from 0.1 to 0.01% of vital cells. Subsequent studies have clarified components contained in extracts which are necessary for gene expression and simultaneously revealed that unnecessary components and inhibitors (for example endogenous nuclease degrading mRNA (RNA 6:1079-1090 (2000)) are contained therein. Thus attempts have been made to eliminate these unnecessary components. However, the conventional method, which comprises using a cell-free extract as a base and eliminating unnecessary components therefrom, suffers from problems that reaction energy is consumed and thus the reaction stops in about 1 hour in protein synthesis when using a batch system. It is pointed out that factors causative of these problems include starvation of nucleotide triphosphates (Biochim. Biophys. Acta. 1293:207-212 (1996), J. Biotechnol. 48:1-8 (1996)), accumulation of small by-products such as triphosphate hydrolyzates formed by endogenous enzymes (Biochemistry 22:346-354 (1983), J. Biol. Chem. 260:15585-15591 (1985)) and energy consumption by factors unnecessary for the transcription/translation reaction (J. Ferment. Bioeng. 84:7-13 (1997), J. Biotechnol. 61:199-208 (1998)).

[0004] The problem of the termination of reaction within a

3

short time can be avoided by continuously supplying a substrate in a transcription/translation reaction system for synthesizing a peptide. However, there arises another problem of poor reproducibility in this case too. This problem has been solved by clarifying the presence of a germ ribosome inactivator (tritin) and a translation initiation inhibitor in studies on a system using wheat germ and employing a means of eliminating these substances from germ (Bio Industry Vol.17, No.5, 20-27 (2000)). However, there still remains another problem that such a system consumes massive energy source wastefully irrespective of translation.

[0005] It was considered that these problems encountering in the conventional methods were caused by the presence of various unknown components in cell extracts which were unnecessary in the transcription or translation reaction but could be hardly eliminated completely. From this viewpoint, an attempt was made to synthesize a peptide *in vitro* by exclusively using enzymes and factors essentially required in the translation (The Journal of Biological Chemistry Vol.252, No.19, 6889-6894 (1997)). For the DNA-directed synthesis of β-galactosidase in this case, use was exclusively made of, in addition to *E. coli* ribosomes, the following 33 components purified from *E. coli* extract as factors and enzymes for the transcription and translation: RNA polymerase, N^{10}-formyltetrahydrofolate Met-tRNA^f transformylase, 20 aminoacyl-tRNA synthetases, IF-1,

4

IF-2, IF-3, EF-Tu, EF-G, RF-1 and/or RF-2, CRP, L and L_α. In this study, however, the target product could be obtained only in a trace amount since only poor information about the translation mechanism and insufficient purification techniques were available in those days.

[0006] Subsequently, Gonza and his co-workers constructed an *in vitro* peptide synthesis system from pre-charged aminoacyl-tRNAs (i.e., having activated amino acid attached thereto) and purified translation factors (Biochem. Biophys. Res. Commun. 126:792-798 (1985)). On the other hand, Pavlov and co-workers reconstructed an *in vitro* translation system using a partially purified aminoacyl-tRNA synthetase mixture with purified translation factors (Archives of Biochemistry and Biophysics Vol.328, No.1, 9-16 (1996)). They also constructed a completely purified *in vitro* translation system using short artificial mRNA (J. Mol. Biol. 273:389-401 (1997)). However, it has never been reported so far as the inventors know that a protein is successfully synthesized from natural mRNA by using a translation system exclusively comprising essential enzymes and factors. In the conventional cell-free peptide synthesis systems and *in vitro* peptide synthesis systems using cell extracts, moreover, troublesome procedures are needed for isolating and purifying a target peptide product from protein components in the reaction system and, therefore, the target peptide can be obtained only at a poor yield.

Summary of the Invention

[0007]   The present invention aims at constructing an efficient protein synthesis system whereby the problem of energy consumption in systems for synthesizing peptides *in vitro* can be overcome, and providing an *in vitro* peptide synthesis system whereby a peptide product can be efficiently isolated from the reaction system at a high purity.

[0008]   The present invention relates to a process for producing a peptide or a peptide derivative by using a reaction system of transcribing a DNA into an RNA and then translating the RNA produced or a reaction system of translating an RNA *in vitro* characterized in that a part or all of protein components constituting the transcription/translation reaction system are labeled with one of a pair of substances adhering to each other and the other substance is used as an adsorbent for capturing said labeled protein components after translating. In this process, a plural number of combinations of the substance used for labeling a part or all of the protein components constituting the reaction system with the substance used as an adsorbent for capturing the labeled protein components may be used in the transcription/translation reaction system.

[0009]   The protein components labeled with one of a pair of

6

the substances adhering to each other are a part or all of factors and enzymes for the transcription or translation reaction. Particular examples of these factors and enzymes include initiation factors, elongation factors, termination factors, aminoacyl-tRNA synthetase, methionyl-tRNA transformylase and RNA polymerase.

[0010] The protein components labeled with one of a pair of the substances adhering to each other are the factors and enzymes for the transcription or translation reaction and other enzymes required in the constitution of the reaction system. Examples of the enzymes required in the constitution of the reaction system other than the factors and enzymes for the transcription or translation reaction include enzymes for regenerating energy in the reaction system and enzymes for hydrolyzing inorganic pyrophosphoric acid formed during the transcription or translation reaction.

[0011] According to the present invention, unnatural peptides carrying unnatural amino acid residues incorporated into desired positions and peptide derivatives such as polysome displays can be produced by using the reaction system for transcribing a DNA into an RNA and then translating the RNA produced or translating an RNA *in vitro* which is free from termination factors.

[0012] In the present invention, the combination of substances mutually interacting in affinity chromatography can be selected

7

from among a combination of a protein or a peptide fragment with a metal ion, a combination of an antigen with an antibody, a combination a protein with a protein or a peptide fragment, a combination a protein with a specific low-molecular weight compound selected from the group consisting of amino acids, DNAs, dyes, vitamins and lectins, a combination of a protein with a saccharide and a combination of a protein or a peptide fragment with an ion exchange resin. Among all, it is favorable to use a combination of histidine tag with a metal chelate such as a nickel complex or a cobalt complex taking advantage of a bond between a protein or a peptide fragment and a metal ion.

[0013] The substances adhering to each other usable in the present invention are not restricted to a combination of substances mutually interacting in affinity chromatography. Use may be made therefor of, for example, substances magnetically adhering to each other too.

[0014] The present invention further relates to a kit of protein components for a reaction system for producing a peptide or a peptide derivative by transcribing a DNA into an RNA and then translating the RNA produced or translating an RNA *in vitro* characterized in that the kit comprises a part or all of protein components constituting the transcription/translation reaction system and that the protein components are selected from the group consisting of enzymes and factors which are labeled with one of a pair of substances adhering to each other.

8

In this kit, the protein components are selected from the factors and enzymes for the transcription or translation reaction and other enzymes required in the constitution of the reaction system. Particular examples of the factors and enzymes for the transcription or translation reaction include initiation factors, elongation factors, termination factors, aminoacyl-tRNA synthetase, methionyl-tRNA transformylase and RNA polymerase. Particular examples of the enzymes required in the constitution of the reaction system other than the factors and enzymes for the transcription or translation reaction include enzymes for regenerating energy in the reaction system and enzymes for hydrolyzing inorganic pyrophosphoric acid formed during the transcription or translation reaction. The kit of protein components according to the present invention may comprise an adsorbent for capturing the protein components labeled with one of a pair of the substances adhering to each other.

[0015] The kit of protein components according to the present invention may comprise combinations, different from each other, of the substance used for labeling a part or all of the protein components constituting the reaction system with the substance used as an adsorbent for capturing the labeled protein components.

Detailed Description of the Invention

9

[0016] The *in vitro* synthesis system of the present invention is a reaction system for synthesizing a peptide by transcribing a DNA into an RNA and then translating the RNA produced or translating an RNA without using cells per se. The term "peptide" as used herein means a substance composed of 2 or more natural or unnatural amino acids attached to each other via peptide bond and involves oligopeptides and polypeptides in its scope. Moreover, proteins having a specific three-dimensional structure of polypeptides fall within this category. The term "RNA" as used herein involves synthetic RNAs and mRNAs in its category, while the term "DNA" as used herein involves synthetic DNAs and cDNAs in its category.

[0017] The *in vitro* synthesis system of the present invention, wherein a DNA is transcribed into an RNA and then the RNA produced is translated or an RNA is translated, is a reaction system existing in prokaryotic cells or eukaryotic cells and consisting of ribosome, factors and enzymes for the transcription or translation reaction, other enzymes required in the constitution of the reaction system, various substrates, buffers and salts. Although the excellent effects of the present invention can be established in a reaction system having been completely reconstituted artificially, the present invention is also applicable to reaction systems wherein some of these constituents are added in the form of a cell extract.

10

[0018] The factors and enzymes for the transcription or translation reaction are not restricted to those originating in prokaryotic cells such as *E. coli* but use can be made of those originating in eukaryotic cells. In case (1) of translating an RNA, these factors and enzymes include initiation factors, elongation factors, termination factors, 20 aminoacyl-tRNA synthetases, and tRNAs attached to natural or unnatural amino acids, and methionyl-tRNA transformuylase is further included in an *E. coli*-origin reaction system. In case (2) of transcribing a DNA into an RNA and then translating the RNA produced, these factors and enzymes include, in addition to those cited in the above case (1), RNA polymerase such as T7RNA polymerase. The translation reaction can be regulated by eliminating the termination factors from the reaction system of the above-described case (1) or (2), as will be discussed hereinafter.

[0019] Examples of the enzymes other than the factors and enzymes for the transcription or translation reaction include enzymes for regenerating energy in the reaction system such as creatine kinase, myokinase and nucleoside diphosphate kinase (NDK), and enzymes for hydrolyzing inorganic pyrophosphoric acid formed during the transcription or translation reaction such as inorganic pyrophosphatase.

[0020] Examples of the various substrates include natural amino acids, unnatural amino acids, nucleotide triphosphates

11

as an energy source, creatine phosphate and formylfolic acid. Nucleotide triphosphates include ATP, GTP, CTP and UTP. ATP and GTP are used in the above-described case (1), while ATP, GTP, CTP and UTP are used in the above-described case (2).

[0021] As the buffer, potassium phosphate buffer (pH 7.3) is usually employed. As the salts, use is usually made of, for example, potassium glutamate, ammonium chloride, magnesium acetate, calcium chloride, putrecine, spermidine and dithiothreitol (DTT). Needless to say, adequate components other than those cited above can be employed too in the reaction system.

[0022] The first characteristic of the present invention resides in that, in a system for synthesizing a peptide by transcribing a DNA into an RNA and then translating the RNA produced or translating an RNA *in vitro,* a part or all of protein components constituting the transcription/translation reaction system are labeled with one of a pair of substances adhering to each other and the other substance is used as an adsorbent for capturing said labeled protein components after translating. Thus, the target peptide can be easily separated from the protein components constituting the reaction system and obtained at an extremely high purity.

[0023] Histidine-tagging has been sometimes employed in producing and purifying individual protein components constituting a reaction system, in particular, factors and

12

enzymes for transcription/translation reaction. For example, it is reported to use histidine-tag in, for example, the production and purification of elongation factors EF-Tu (Eur. J. Biochem. 210:177-183 (1992)), EF-G (Cell 92: 131-139 (1998)) and EF-Ts (Archives of Biochemistry and Biophysics 348:157-162 (1997)), the production and purification of a termination factor RF2 (Proc. Natl. Acad. Sci. USA 95:8165-8169 (1998)), and the production and purification of phenylalanyl-tRNA synthetase (Protein Expression and Purification 8: 347-357 (1996)). In these cases, however, the production and purification were carried not to construct an *in vitro* peptide synthesis system but merely to examine the functions or properties of individual proteins.

[0024] To separate a protein or a protein derivative produced by expressing a gene in a transformant, such as *E. coli*, affinity chromatography with the use of, for example, a combination of histidine tag with a nickel column, a combination of glutathione S-transferase with a glutathione-Sepharose resin column or a combination of an epitope tag with an antibody has been used. In such a case, it has been a practice to incorporate a residue capable of selectively binding to the adsorption column into the target peptide. In cell-free systems with the use of marketed cell extracts, use is made of a vector for incorporating histidine tag into a target peptide. In such a case, therefore, the product is obtained in the form of a fused

13

protein of the target peptide with the histidine tag which should be enzymatically cut off from the peptide after synthesizing.

[0025] On the contrary to these conventional methods, the present inventors have introduced one of a pair of substances adhering to each other not into the target peptide but protein components constituting the *in vitro* peptide synthesis system, based on a novel finding that the transcription or translation reaction can proceed even though the factors and enzymes for the transcription or translation and other enzymes are labeled with one of a pair of the substances adhering to each other.

[0026] The combination of a pair of the substances adhering to each other to be used in the present invention may be an arbitrary one, so long as the transcription or translation reaction is not disturbed thereby. Although the adhesion of these substances to each other may be either reversible or irreversible, it is preferable to use a pair of substances which irreversibly adhere to each other. This is because the protein components constituting the reaction system can be repeatedly used in such a case.

[0027] As an example of the combination of the substances adhering to each other, citation may be made of a combination of an adsorption column with a substance capable of selectively binding to the adsorption column. Typical examples thereof include substances mutually interacting in affinity

14

chromatography. For example, a metal complex such as a nickel or cobalt complex serves as a ligand of an adsorption column while histidine tag serves as a substance capable of selectively binding to the adsorption column. Moreover, use can be made of combinations of various ligands with substances capable of selectively binding thereto as will be discussed hereinafter, so long as the reaction is not disturbed thereby. That is to say, the combination of the substances mutually interacting in affinity chromatography usable in the present invention can be selected from among, for example, a combination of a protein or a peptide fragment with a metal ion, a combination of an antigen with an antibody, a combination of a protein with a protein or a peptide fragment, a combination of a protein with a specific low-molecular weight compound selected from the group consisting of amino acids, DNAs, dyes, vitamins and lectins, a combination of a protein with a saccharide and a combination of a protein or a peptide fragment with an ion exchange resin.

[0028] The substances adhering to each other usable in the present invention are not restricted to a combination of substances mutually interacting in affinity chromatography but can be arbitrarily selected depending on the purpose. For example, use may be made therefor of substances magnetically adhering to each other. As an example thereof, a combination of a magnetic bead-labeled protein with a magnet may be cited.

15

In this case, protein components constituting the peptide synthesis system, which have been individually labeled with the magnetic beads, can be adsorbed by the magnet and thus captured.

[0029] In the present invention, the adsorbent is used in the form of, for example, a column, a matrix, a filter or a bead. Alternatively, it may be fixed to a carrier (support), if desired. To fix the adsorbent to the carrier, an appropriate means can be selected from among known techniques depending on the properties of the adsorbent.

[0030] There are a plural number of combinations of the substance for labeling a part or all of the protein components constituting the reaction system with the substance used as the adsorbent for capturing the thus labeled protein components. It is possible to use such combinations differing from each other in a single reaction system. It may be rather considered as favorable to select the most suitable labels for respective protein components and then select adsorbents appropriate for these labels.

[0031] Since the factors and enzymes for the transcription/translation reaction system and other enzymes are labeled with one of a pair of the substances adhering to each other in the present invention, the protein components constituting the reaction system can be obtained each in a highly pure state and the reaction system is not contaminated with any unknown and unnecessary or inhibitory components.

16

Thus, the reaction system can be established and, consequently, the reaction efficiency can be largely elevated. In addition, it becomes possible to quickly separate the target peptide from these reaction constituents after synthesizing the peptide. In the conventional cell-free systems, a peptide formed by the reaction is purified by extraction. It is therefore needed to select an appropriate purification procedure in each case depending on the physical and chemical properties of the reaction product. In the present invention, in contrast thereto, the components constituting the reaction system are eliminated by using the adsorbent and the reaction product is thus purified. Accordingly, it is theoretically possible to apply the same purification procedure to any reaction products regardless of the physical and chemical properties thereof. In addition, the target peptide thus obtained has a very high purity.

[0032] According to the present invention, furthermore, the components of the reaction system can be surely controlled, which makes it possible to establish a reaction system free from termination factors. Owing to this characteristic, the present invention enables the construction of polysome displays of various types, thereby broadening the application range of the *in vitro* peptide synthesis systems. More particularly speaking, ternary complexes (polysome displays) composed of peptide, RNA and ribosome can be obtained by expressing various

17

DNAs or RNAs in the termination factor-free *in vitro* peptide synthesis system according to the present invention. By separating such a polysome display from other complexes with the use of the peptide as a target, the target peptide and RNA can be obtained at the same time. By treating the product with termination factors, the corresponding RNA can be obtained. In this case, the peptide, which has been cut off from the ribosome, and RNA corresponding to it can be easily isolated by treating with termination factors labeled with one of a pair of the substances adhering to each other. For example, a corresponding DNA can be obtained from an isolated polysome display by using the RT-PCR method, as described in Current Opinion in Biotechnology 9:534-548 (1998). By decomposing this polysome display with EDTA, an RNA can be obtained. In particular, it is technically advantageous that an RNA or a DNA corresponding to a selected target peptide can be easily obtained in random expression. Although semirandom expression of DNAs and RNAs in a cell-free system with the use of a rabbit reticulocyte kit is already known, complicated procedures are needed in this case (WO91/05058).

[0033] By surely controlling the components constituting the reaction system, it becomes possible to synthesize a peptide having an unnatural amino acid residue by the *in vitro* peptide synthesis system. Namely, a peptide having an unnatural amino acid residue can be synthesized by using the production process

according to the present invention as follows. A suppressor tRNA charged by an unnatural amino acid residue and corresponding to a termination codon differing from the C-terminal termination codon is added to the reaction system. Then a DNA or an RNA, which has been modified by inserting a termination codon corresponding to the suppressor tRNA into a position for the incorporation of an unnatural amino acid residue, is transcribed or translated to thereby give a peptide having the unnatural amino acid residue incorporated thereinto. Speaking in greater detail, the synthesis can be carried out as follows. To incorporate the unnatural amino acid residue, one of termination codons (UAA, UAG and UGA) such as UGA or UAG is inserted into a desired position within the open reading frame (ORF) and UAA is employed for terminating the translation. Subsequently, a suppressor tRNA carrying an anticodon to UGA and/or UAG is formed by *in vitro* transcription and charged by the unnatural amino acid. Then the production process according to the present invention is performed by using this RNA and suppressor tRNA as described above to translate the RNA. Thus, a peptide having the unnatural amino acid residue site-specifically inserted therein can be synthesized. Alternatively, the peptide can be synthesized by separately synthesizing the corresponding DNA followed by transcription and translation.

[0034] Use of a reaction system obtained by eliminating

termination factors from the above-described reaction system
makes it possible to obtain a polysome display composed of
peptide, mRNA and ribosome which carries one or more unnatural
amino acid residues at desired position(s). By treating this
polysome display with termination factors labeled with one of
a pair of substances adhering to each others as in the above
case, the peptide, which has been cut off from the ribosome,
and the RNA corresponding to it can be easily isolated. For
example, a corresponding DNA can be obtained from a polysome
display by using the RT-PCR method as described in Current
Opinion in Biotechnology 9:534-548 (1998). By decomposing
this polysome display with EDTA, it is also possible to obtain
the RNA.

[0035] Accordingly, the peptide derivatives produced by the
process of the present invention involve polysome displays and
unnatural peptides having unnatural amino acid residues at
desired positions.

[0036] The process for producing a peptide or a peptide
derivative according to the present invention can be performed
by using a batch system in a conventional manner. Alternatively,
it may be carried out by using various already known or usual
methods such as a flow method, wherein materials including the
substrates are continuously supplied or the reaction product
is occasionally withdrawn, or a dialysis method (see, for
example, Jpanese patent publication 110236/1995,

Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic acids and Enzymes) Vol.44, No.4, 598-605 (1999), Current Opinion in Biotechnology 9:534-548 (1998)).

[0037] Next, the present invention will be illustrated in greater detail by reference to the following examples. However, it should be understood that the invention is not construed as being restricted thereto.

(1)   Peptides and peptide derivatives which can be produced

       by the invention

[0038] According to the production process of the present invention, natural peptides and unnatural peptides of any types can be produced. That is to say, use of the process of the present invention makes it possible to produce peptides which would be digested by proteases originating in host cells such as dihydrofolate reductase (DHFR), lysozyme (originating in λ-phage) and green fluorescent proteins (GFPs), and peptides showing toxicity on host cells. The term "natural peptides" as used herein means peptides composed of 20 natural amino acids used in genetic codes, while peptides containing other α-amino acids are called "unnatural peptides".

[0039] Moreover, ternary complexes (polysome displays) composed of peptide, RNA and ribosome can be easily obtained by using a termination factor-free reaction system in the production process of the present invention.

[0040] Examples of unnatural peptides which can be produced by the process according to the present invention include peptides having modified natural amino acids, modified non-charged amino acids, modified acidic amino acids, modified basic amino acids, non-α-amino acids, amino acids with ϕ,ϕ-angle displacement, and amino acids having functional groups selected from the group consisting of nitro, amidine, hydroxylamine, quinone, aliphatic compounds, and cyclic and unsaturated hydrocarbyl groups. There have been already known processes for synthesizing these peptides in cell-free protein synthesis systems with the use of cell extracts (see, for example, JP-W-Hei-4-504651/WO90/05785). As a particular example of these unnatural peptdies, DHFR having a protected cysteine residue inserted into an appropriate position, which would not occur in nature, may be cited. Further citation may be made of peptides having an unnatural amino acid residue such as p-fluorophenylalanine, p-nitrophenylalanine or homophenylalanine incorporated into a desired position. It is already known that β-lactamase variants having these 3 unnatural amino acid residues incorporated as a substitute for phenylalanine at the 66-position show sufficient enzyme activity (Bio Industry 8:749-759 (991)). These unnatural peptides can be easily produced by the process according to the present invention too.

(2) Ribosome

22

[0041] Ribosome is a particle where peptides are synthesized. It binds to mRNA and coordinates aminoacyl-tRNA to the A-position and formylmethionyl-tRNA or peptidyl-tRNA to the P-position, thereby forming a peptide bond (Science 289:920-930 (2000)). In the present invention, any ribosome can be used regardless of the origin, so long as it has the function as described above. Although *E. coli* ribosome is usually employed, use can be made of eukaryotic ribosomes too. It is preferable in the present invention to use *E. coli* ribosome, for example, those obtained from *E. coli* A19 strain or MRE600 strain.

(3) Factors and enzymes for the transcription or translation to be used in the in vitro peptide synthesis system according to the present invention

(3-1) Initiation factors

[0042] Initiation factors means factors which are essentially required in the formation of an initiation complex in the process of peptide synthesis or remarkably promote it. IF1, IF2 and IF3 are known as initiation factors originating in *E. coli* (Biochemistry 29:5881-5889 (1990)). IF3 promotes the dissociation of ribosome into 30S and 50S subunits (i.e., the step required for initiating translation) and hinders the insertion of tRNAs other than formylmethionyl-tRNA into the P-position in the step of forming the initiation complex. IF2 binds to formylmethionyl-tRNA and transfers the formylmethionyl-tRNA to the P-position of 30S subunit, thereby

23

forming the initiation complex. IF1 potentiates the functions of IF2 and IF3. In the present invention, it is preferable to use *E. coli*-origin initiation factors, for example, those obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic initiation factors.

(3-2) Elongation factors

[0043] An elongation factor EF-Tu is classified into 2 types, i.e., GTP and GDP types. EF-Tu of the GTP type binds to aminoacyl-tRNA and transfers it to the A-position of ribosome. When EF-Tu is released from ribosome, GTP is hydrolyzed into GDP (EMBO J. 17:7490-7497 (1998)). Another elongation factor EF-Ts binds to EF-Tu of the GDP type and promotes the conversion of it into the GTP type (Archives of Biochemistry and Biophysics 348:157-162 (1997)). Another elongation factor EF-G promotes translocation following the peptide bond formation in the process of peptide chain elongation (Nature Structural Biology 6:643-647 (1999), FEMS Microbiology Reviews 23:317-333 (1999)). In the present invention, it is preferable to use *E. coli*-origin elongation factors, for example, those obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic elongation factors.

(3-3) Termination factors

[0044] Termination factors are essentially required in terminating protein synthesis, releasing the translated peptide chain and recycling ribosome for the initiation of the

24

subsequent mRNA translation. When a protein is synthesized in a termination factor-free reaction system, the reaction stops before the termination codon and thus a stable ternary complex (polysome display) composed of ribosome, peptide and mRNA can be easily formed. An unnatural amino acid is incorporated into a peptide chain by eliminating either RF1 or RF2 from the reaction system. That is to say, an unnatural amino acid is incorporated at a high efficiency into the UAG codon in case of eliminating RF1 or into the UGA codon in case of eliminating RF2.

[0045] When a termination codon (UAA, UAG or UGA) is located at the A-position of ribosome, termination factors RF1 and RF2 enter the A-position and promote the dissociation of the peptide chain from peptidyl-tRNA at the P-position. RF1 recognizes UAA and UAG among the termination codons, while RF2 recognizes UAA and UGA. Another termination factor RF3 promotes the dissociation of RF1 and RF2 from ribosome after the dissociation of the peptide chain by RF1 and RF2. Ribosome recycling factor (RRF) promotes the dissociation of tRNA remaining at the P-position after the protein synthesis and the recycling of ribosome for the subsequent protein synthesis. In the present invention, RRF is referred to as one of termination factors. The functions of these termination factors RF1, RF2, RF3 and RRF are described in EMBO J. 16:4126-4133 (1997) and EMBO J. 16:4134-4141 (1997). In the present invention, it is

25

preferable to use *E. coli*-origin termination factors, for example, those obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic termination factors.

(3-4) Aminoacyl-tRNA synthetase

[0046] Aminoacyl-tRNA synthetase is an enzyme by which an amino acid is covalently bonded to tRNA in the presence of ATP to thereby synthesize aminoacyl-tRNA (RNA 3:954-960 (1997), Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids and Enzymes) 39: 1215-1225 (1994)). In the present invention, it is preferable to use *E. coli*-origin aminoacyl-tRNA synthetase, for example, one obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic aminoacyl-tRNA synthetases.

(3-5) Methionyl-tRNA transformylase

[0047] N-Formylmethionine (fMet) carrying a formyl group attached to the amino group at the end of methionine serves as the initiation amino acid in a prokaryotic protein synthesis system. This formyl group is attached to the methionine in methionyl-tRNA by methionyl-tRNA transformylase (MTF). Namely, methionyl-tRNA transformylase transfers the formyl group in N^{10}-formyltetrahydrofolate to the N-terminus of methionyl-tRNA corresponding to the initiation codon, thereby giving formylmethionyl-tRNA (Proc. Natl. Acad. Sci. USA 96:875-880 (1999)). The formyl group thus attached is recognized by IF2 and thus acts as an initiation signal for

protein synthesis. Although MTF does not occur in the synthesis system in eukaryotic cytoplasm, it is present in the synthesis systems in eukaryotic mitochondria and chloroplast. In the present invention, it is preferable to use *E. coli*-origin methionyl-tRNA transformylase, for example, one obtained from *E. coli* K12 strain.

(3-6) RNA polymerase

[0048] It is known that RNA polymerase, which is an enzyme transcribing a DNA sequence into an RNA, occurs in various organisms. As an example thereof, citation may be made of T7RNA polymerase originating in T7 phage which is an enzyme binding to a specific DNA sequence called T7 promoter and then transcribing the downstream DNA sequence into an RNA. The present inventors attached His-tag to the N-terminus of this T7RNA polymerase and expressed it as a fused protein in a large amount in *E. coli* BL21 strain. Then, they purified the expression product by affinity chromatography with the use of a nickel column. The His-tagged T7RNA polymerase thus obtained is a novel one. In addition to T7RNA polymerase, various RNA polymerases are usable in the present invention. For example, commercially available T3RNA polymerase and SP6RNA polymerase can be used.

(3-7) Aminoacyl-tRNA attached to unnatural amino acid

[0049] By incorporating amino acid residues, other than 20 amino acids constituting natural proteins, into proteins, it

27

is possible to improve the functions inherent to the proteins or to impart new useful functions or characteristics to the proteins. Aminoacyl-tRNA attached to an unnatural amino acid can be produced by synthesizing a 3'-terminal CA-deficient suppressor tRNA via *in vitro* transcription and ligating it to chemically synthesized aminoacyl-pCpA having the unnatural amino acid by using RNA ligase (Baiosaiensu to Indasutori (Bioscience and Industry) 47:16-24 (1989)).

(4)   Enzymes required in the constitution of the reaction system other than the factors and enzymes for the transcription or translation to be used in the *in vitro* peptide synthesis system according to the present invention

(4-1) Enzymes for regenerating energy in the reaction system

[0050] Examples of the enzymes of this type include creatine kinase, myokinase and nucleoside diphosphate kinase (NDK). Creatine kinase, which is also called creatine phosphokinase (CPK), catalyzes transfer of phosphate group from ATP to creatin. Myokinase, which is also called adenylate kinase, participates in the regeneration of ATP from ADP and the simultaneous formation of AMP. NDK catalyzes γ-phosphate group transfer between nucleoside diphosphate and nucleoside triphosphate. In the present invention, it is preferable to use these enzymes originating in *E. coli*, for example, those obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic

28

enzymes.

(4-2) Enzymes for hydrolyzing inorganic pyrophosphoric acid

formed during the transcription or translation reaction

[0051] As an example of the enzymes of this type, inorganic pyrophosphatase may be cited. In the present invention, it is preferable to use *E. coli*-origin enzymes, for example, those obtained from *E. coli* K12 strain. However, it is also possible to use eukaryotic enzymes.

[0052] Among the constituents of the reaction system as described in the above (3) and (4), protein components are expressed in a large amount in *E. coli* (for example, commercially available *E. coli* BL21 strain) in the form of fused proteins (for example, His-tagged proteins) labeled at the N- or C-terminus with one of a pair of the substances adhering to each other as will be described in greater detail hereinafter. Then these proteins thus expressed are purified by using a nickel column connected to an adsorbent containing the other substance such as fast protein liquid chromatography (FPLC) and then supplied to the reaction system. In addition to *E. coli*, it is possible to express these proteins in animal cells, yeasts, *Bacillus subtilis* or the like. Alternatively, it is possible to produce these proteins by using an *in vitro* peptide synthesis system.

(5) Label and adsorbent, i.e., a pair of substances adhering
     to each other

[0053] In the present invention, all or a part of the protein
components constituting the reaction system as described in the
above (3) and (4) are labeled with one of a pair of substances
adhering to each other and the thus labeled protein components
are captured by using the other substance as an adsorbent to
thereby isolate the target peptide formed in the reaction system.
As typical examples of a pair of the substances adhering to each
other, substances mutually interacting in affinity
chromatography can be cited. However, any pair of substances
adhering to each other are usable in the present invention
without restriction to substances mutually interacting in
affinity chromatography, so long as these substances can be used
in capturing the protein components.

[0054] Proteins exert physiological effects via specific
mutual interactions with certain substances. Adsorption
chromatography which is carried out by taking advantage of such
a specific interaction (affinity) between a protein and a
certain substance (ligand) is called affinity chromatography.
Examples of a combination of substances adhering specifically
to each other include a protein or a peptide fragment with a
metal ion or a chelate compound, an antigen with an antibody,
a cytokine or a hormone with a receptor, and an enzyme with a
substrate or an inhibitor. Furthermore, specific amino acids,

30

DNAs, dyes, vitamins, lectins and the like mutually bind to proteins having affinities therefor respectively.

[0055] One of the substances of such a combination is fixed as a ligand to a carrier or a support to form an adsorbent. Then materials labeled with the other substance (the protein components constituting the reaction system in the case of the present invention) are passed therethrough. Thus, the label specifically binds to the ligand. Affinity chromatography based on this specific binding has been commonly employed as a means of purifying proteins. Various carriers have been marketed by a number of manufacturers, which makes this means highly available. In purifying a protein using an antigen-antibody reaction, for example, use is made of a combination of an antigen determinant (an epitope) having a known structure with an antibody specific to the epitope. There have been marketed various combinations of vectors and adsorbents for carrying out this means. In a preferred embodiment of the present invention, use is made of a combination of such substances adsorbing to each other which are employed in affinity chromatography. In preparing the labeled protein components to be used in the present invention, the label is useful in the purification. It is also possible to label the protein components with a plural number of labels at the same time. In this case, it is possible that the label usable for the purification in the production process is cut

off while other labels are used for the separation of the target

product formed in the *in vitro* peptide synthesis system.

[0056]   Now, embodiments of the present invention will be

illustrated by citing some examples of the combination of the

substances adhering to each other.   However, it is to be

understood that the present invention is not construed as being

restricted these examples.

(5-1) Process with the use of binding of protein or peptide

         fragment to metal ion or chelate compound

A. His tag with metal complex such as nickel complex or cobalt

   complex

[0057]   It has been a practice to purify a protein by using the

binding of His tag to a metal complex such as a nickel complex

or a cobalt complex.   Namely, His tag is attached to a DNA to

be expressed to give a fused protein having His tag.   Then this

fused protein is captured and purified by using a column having,

for example, a nickel complex, cobalt complex, a copper complex

or a zinc complex.   Then the protein can be eluted from the

column with an eluent containing imidazole (see, for example,

Tanpakushitsu Jikken Noto (Protein Experiment Note) (I), on and

after p.139, Chap. 5: 1. His-Tag Tanpakusthitu no Hatugen to

Seisei (Expression and Purification of His-Tag Protein),

Yasumitsu and Wakui, published by Yodosha; J. A. Bornhorst and

J. J. Falke, Purification of Proteins Using Polyhistidine

Affinity Tags. Methods in Enzymology 326:245-254, (2000);

Proteins 41:144-53 (2000), FEMS Microbiol. Lett. 188:147-51

(2000); and J. Bacteriol. 182: 4304-9 (2000)). These

combinations are usable in the present invention.

[0058] To express genes encoding His-tagged protein

components, it is known to use, for example, *E. coli* (M.W.Van

Dyke, M.Sirito, and M.Sawadogo, Gene 111:95, 1992),

Saccharomyces cerevisiae (D.C.Kaslow and J.Shiloach,

Bio/Technology 12:494, 1994), mammalian cells (R.Janknecht and

A.Nordheim, Gene 121:321, 1992), and baculovirus-infected

insect cells (A.Kuusinen, M.Arvola, C.Oker-Blom, and

K.Keinänen, Eur. J. Biochem. 233:720, 1995). These cells may

be optionally used in the present invention.

[0059] The following protocol roughly shows an example of the

process of the purification of a His-tagged protein component

by using His tag and a nickel column. There have been known

a number of variations of this process and an appropriate one

may be selected therefrom.

1. Attach a His tag consisting of 6 His residues to the

N-terminus of a target protein by a genetic engineering

technique to thereby give a fused protein.

2.Disrupt cells expressing the tagged protein by

sonication on ice and suspend the disrupted cells in loading

buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) .

3. Centrifuge the cell lysate (30,000g, 4°C, 30 min).

33

4. Add 50% Ni^{2+}-NTA slurry (Qiagen) pre-equilibrated in ice-cold loading buffer to the supernatant. Stir at 4°C for 1 hr.

5. Load the resin onto a column. Wash the column with 20 column volumes of loading buffer at 4°C.

6. Wash the column with 20 column volumes of loading buffer (containing 10 mM imidazole, pH 8.0) at 4°C.

7. Elute the target protein with a 20 column volumes of loading buffer under an imidazole concentration gradient of 10 to 250 mM in the loading buffer. Collect 1 ml fractions and identify the target protein by SDS-PAGE.

B. Thioredoxin with phenylarsine oxide (PAO)

[0060] In this process, a fused protein composed of a target protein with thioredoxin is formed and adsorbed by a PAO-fixed agarose gel (ThioBond™ resin, Invitrogen) by taking advantage of binding of thioredoxin to PAO followed by elution with β-mercaptoethanol (β-ME) (A. Alejo, R. J. Yanez, J. M. Rodriguez, E. Vinuela, and M. L. Salas, African Swine Fever Virus trans-Prenyltransferase, The Journal of Biological Chemistry 272: 9417-9423, 1997). This process is also usable in the present invention.

[0061] The following protocol roughly shows an example of the process of the purification of a protein by using this process.

1. Dilute overnight culture of transformed cells (*E. coli*/ vector pTrxFus, Invitrogen) 20-fold in RM medium (0.6 %

Na_2HPO_4, 0.3 % KH_2PO_4, 0.05 % NaCl, 0.1 % NH_4Cl, 2 % casamino acids, 0.0095 % $MgCl_2$) containing 100 µg/ml (final concentration) of ampicillin and incubate at 30°C.

2. After incubating to A_{550}=0.5, add 100 µg/ml (the final concentration) of tryptophan to induce the expression of the fused protein. Then continue the incubation at 34°C for additional 2 hr.

3. Harvest the cells by centrifugation. Suspend the cell pellet in 5 ml of running buffer [100 mM Tris-HCl (pH 7), 150 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol]. Then disrupt the cells by sonication.

4. Centrifuge the cell suspension (10,000g, 15 min) and collect the supernatant.

5. Incubate the supernatant with 2 ml of ThioBond™ resin at 4°C for 60 min to bond the fused protein contained in the supernatant to the resin.

6. Pack a column with the slurry and wash with 30 column volumes of running buffer [100 mM Tris-HCl (pH 7), 150 mM NaCl, 1 mM EDTA, 20 mM β-mercaptoethanol].

7. Elute the target protein with the running buffer under a β-mercaptoethanol gradient.

(5-2) Process with the use of binding of antigen or antigen
    fragment (epitope tag) to antibody

A. T7-tag and monoclonal antibody specific to T7-tag

[0062] T7-Tag is a sequence consisting of 11 amino acids of

gene 10 originating in phage T7. A combination of T7-tag with an antibody against it is employed in a means of purifying protein. Namely, this process comprises attaching a DNA sequence encoding T7-tag to a gene, then expressing the target protein, and capturing and purifying the T7-tagged fused protein thus obtained by using a monoclonal antibody specific to T7-tag as an adsorbent. As an example of the adsorbent, T7-Tag Antibody Agarose is marketed (Novagen). Citric acid is used as an eluent. This combination is also usable in the present invention. See R. Deora, T. Tseng, and T. K. Misra, Alternative Transcription Factor σ^{SB} of *Staphylococcus aureus*: Characterization and Role in Transcription of the Global Regulatory Locus sar. Journal of Bacteriology 179:6355-6359, 1997.

[0063] The following protocol roughly shows an example of the process of the purification of a T7-tagged protein component. There have been known a number of variations of this process and an appropriate one may be selected therefrom.

1. Grow transformed cells E. coli/vector pET (Novagen) in 2xYT medium (1.6 % Bacto Trypton, 1 % yeast extract, 0.5 % NaCl, 0.4 % glucose) containing 20 µg/ml of chloramphenicol and 30 µg/ml of kanamycin.

2. After incubating to $A_{600}=0.6$, add IPTG to induce the expression of the target protein. Then continue the incubation for additional 2 hr.

3. Harvest the cells by centrifugation. Suspend the cell pellet in 10 ml ice-cold T7-Tag bind/wash buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 1 % Tween-20, 0.02 % sodium azide (pH 7.3)).

4. Disrupt the cells by sonication on ice until the suspension shows no viscosity any more.

5. Centrifuge (39,000g, 20 min) to eliminate cell debris. Filter the supernatant through a 0.45 μm filter membrane.

6. Apply the cell extract into a T7-Tag Antibody Agarose column (Novagen) pre-equilibrated with T7-Tag bind/wash buffer. Wash the column with the same buffer to eliminate nonspecifically bonded proteins.

7. Elute the target protein with elution buffer (0.1 M citric acid (pH 2.2)).

B. Process of using binding of FLAG peptide tag™ (Sigma) to anti-FLAG antibody™ (Sigma)

[0064] FLAG peptide tag™ (Sigma, and so on), which is a peptide consisting of 8 amino acids, is used in purifying proteins as a so-called epitope tag together with an antibody against it. Namely, a protein having the FLAG peptide tag at the N-terminus is constructed and captured by a FLAG antibody column. FLAG peptide is used in elution. See P. J. Woodring and J. C. Garrison, Expression, Purification, and Regulation of Two Isoforms of the Inositol 1,4,5-Trisphosphate 3-Kinase. The Journal of Biological Chemistry 272: 30447-30454, 1997.

[0065]   The following protocol roughly shows an example of this process which may be appropriately modified.

1. Collect cells (host: B31 cell (Rat-1 fibroblast cell line), vector: pDouble-Trouble (pDT) mammalian expression vector) showing the expression of the target protein by centrifugation and homogenize in 8 ml of hypotonic lysis buffer containing protease inhibitors (10 µg/ml calpain inhibitors I and II, 100 µg/ml Pefabloc, 2.5 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µg/ml bacitracin, 20 µg/ml benzamidine).

2. Centrifuge (2,000 g) to eliminate cell debris and nucleic acids and collect the supernatant.

3. Further centrifuge and apply the cell extract thus obtained to 1 ml of a FLAG antibody column. Wash the column with 35 ml of TBSC (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 % (v/v) CHAPS).

4. Elute the target protein with 5 ml of TBSC containing 200 µg/ml of the FLAG peptide.

C. Protein A and IgG

[0066]   In this process, use is made of the binding of Staphylococal Protein A (SPA) to antibody IgG thereof. A fused protein composed of a target protein with SPA is captured by using an IgG Sepharose column. Buffer with a low pH value is used in elution. See B. Nilsson and L. Abrahmsen, Fusion to Staphylococcal Protein A. Methods in Enzymology 185: 144-161, 1990.

[0067]   The following protocol roughly shows an example of this process which may be appropriately modified.

1. Add overnight culture of transformed cells (host: *E. coli* or *S. aureus*, vector: pRIT 20 or pRIT30 series) to 25 ml of LB medium (LB medium + 0.1%(w/v) glucose, 250 mg/l ampicillin) and incubate at 37°C for 4 hr.

2. Harvest the cells by centrifugation (10,000g, 5°C, 20 min) and filter the supernatant through a 0.45 μm filter membrane.

3. Apply the filtrate to 5 ml of an IgG Sepharose column pre-equilibrated with TST (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05 % (v/v) Tween 20).

4. Wash the column successively with 15 ml aliquots of TST twice and 5 ml of 1 mM ammonium acetate.

5. Elute the protein with 1 ml of 0.5 M ammonium acetate (pH 3.3).

D. Protein and monoclonal antibody

[0068]   There is known a method of purifying cyclic nucleotide-gated (CNG) channel, which is a protein originating in bovine retina, by using a monoclonal antibody PMc 6E7 (N-terminal domain of α subunit: 63-kDa polypeptide) (R. S. Molday and L. L. Molday, Purification, Characterization, and Reconstitution of Cyclic Nucleotide-Gated Channels. Methods in Enzymology 294: 246-260, 1999). Sepharose 2B (Pharmacia) carrying the monoclonal antibody fixed thereon is used as an

adsorbent. To elute the target protein, use is made of 6E7 competing peptide which is a peptide competitively binding to the monoclonal antibody and having an amino acid sequence Ser- Asn- Lys- Glu- Gln- Glu- Pro- Lys- Glu- Lys- Lys- Lys- Lys- Lys. This combination is also usable in the present invention.

[0069] The following protocol shows an example of the process for purifying the bovine retina-origin protein with the use of the monoclonal antibody.

1. Collect rod outer segment (ROS) fractions from a bovine retina homogenate by centrifugation under a 30 to 50% sucrose density gradient (20 mM Tris-acetate (pH 7.4), 10 mM glucose, 1 mM $MgCl_2$; 82,500 g, at 4°C for 45 min).

2. Dilute the ROS fraction in 5 volumes of homogenizing buffer (20 % (w/v) sucrose, 20 mM Tris- acetate (pH 7.4), 10 mM glucose, 1 mM $MgCl_2$) and centrifuge (20,000 g, 4°C, 20 min).

3. Re-suspend the ROS pellet in 8 ml of homogenizing buffer to give a crude ROS extract.

4. Suspend ROS in 10 volumes of hypotonic lysis buffer (10 mM HEPES- KOH (pH 7.4), 1 mM EDTA, 1 mM DTT) and centrifuge (20,000g, 10 min).

5. Suspend the membrane pellet in the same buffer and repeatedly wash twice.

6. Suspend the pellet in 10 mM HEPES-KOH (pH 7.4).

7. Add the ROS membranes to CHAPS (3 - [3- (Cholamidopropyl) dimethylammonio] -1- propane sulfonate)

solubilization buffer [10 mM HEPES- KOH (pH 7.4), 10 mM $CaCl_2$, 0.15 M KCl, 18 mM CHAPS, 2 mg/ml asolectin (soybean phosphatidylcholine, type IV-S; Sigma) protease inhibitor (0.1 mM diisoprophylfluorophosphate, 5 μg/ml aproteinin, 1 μg/ml leupeptin, 2 μg/ml pepstatin or 20 μM Pefabloc SC)] and stir slowly.

8. Centrifuge (27,000 g, 4°C, 30 min) to eliminate cell debris.

9. Apply 20 ml of the solubilized ROS membrane to a Sepharose 2B column having PMc 6E7(antibody) fixed thereon. Wash with 10 volumes of CHAPS column buffer (10 mM HEPES- KOH (pH 7.4), 1 mM $CaCl_2$, 0.15 M KCl, 12 mM CHAPS, 2 mg/ml asolectin).

10. Elute the target protein with CHAPS column buffer containing 0.1 mg/ml of 6E7 competing peptide.

(5-3) Process with the use of binding of protein to protein or peptide fragment

A. Strep-Tag and streptavidin

[0070] There has been known a process for affinity purification of a protein having Strept-tag which is an oligopeptide having an affinity for streptavidin (see, for example, A. Skerra and T. G. Schmidt, Use of the Strep-Tag and Streptavidin for Detection and Purification of Recombinant Proteins. Methods in Enzymology 326: 271-311(2000) 、 BioTechniques 28: 338-344 (2000)). This combination is also usable in the present invention.

41

[0071] In this process with the use of the binding of Strep-tag to streptavidin, use is made as the Strep-tag, for example, Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly or Asn-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (Strep-tag II). A Strep-tagged protein such as DHFR (dihydrofolate reductase) is synthesized in a cell-free system. Then it is purified by adsorbing by fixed streptavidin or Strep Tactin. As an eluent, desthiobiotin is employed.

[0072] The following protocol shows an example of the process of the production of a Strep-tagged protein component with the use of Strep-tag and streptavidin. There have been known a number of variations of this process and an appropriate one may be selected therefrom.

1. Add overnight culture of transformed *E. coli* cells (using pASK-IBA vector) to 2 l of fresh LB medium (containing ampicillin at final concentration of 100 µg/ml) and incubate to OD_{550}=0.5 under shaking (200 rpm) at 22°C.

2. To induce gene expression, add 200 µl of 2 mg/ml anhydrotetracycline-dimethylformamide(DMF) solution. Then continue the incubation for additional 3 hr.

3. Harvest the cells by centrifuging (4,200g, 4°C, 12 min). Suspend the cells in 20 ml of buffer P (100 mM Tris-Cl (pH 8.0), 500 mM sucrose, 1mM Na_2EDTA) and incubate on ice for 30 min.

4. Eliminate spheroplast by centrifuging (27,000 g, 4°C, 15 min).

5. Dialyze the thus obtained periplasma faction against 2 l of buffer (100 mM Tris-Cl (pH 8.0), 1 mM Na$_2$EDTA) overnight.

6. Equilibrate a StrepTactin Sepharose column with buffer W. Apply the protein solution to the column by using a system provided with a peristaltic pump, an UV detector (A$_{280}$) and a fraction collecter.

7. Wash the column with buffer W until A$_{280}$ attains the base line.

8. Elute the target protein with buffer W containing 2.5 mM of desthiobiotin.

B. S-peptide and S-protein

[0073] It is known that the protein fragment of ribonuclease S (S-protein) tightly binds to its S-peptide fragment (S-peptide) reversibly. Protein can be purified with the use of this binding. Namely, an S-tagged protein (i.e., having S-peptide attached thereto) can be captured by agarose having S-protein fixed thereto. Elution is carried out by cleaving the bond between S-tag and S-protein with, for example, 3M guanidinium thiocyanate; 0.2 M potassium citrate buffer, pH 2; 3 M MgCl$_2$ (R. T. Raines, M. McCormick, T. R. V. Oosbree, R. C. Mierendorf, The S · Tag Fusion System for Protein Purification. Methods in Enzymology 326:362-376, 2000).

[0074] The following protocol roughly shows an example of this process which may be optionally modified.

1. Add 2 ml of an S-protein agarose slurry (Novagen) to

an extract of cells expressing the target protein (host: bacteria, insect, mammalian, vector: pET, pBAC (Novagen) and thoroughly stir at room temperature for 30 min.

2. Centrifuge (500g, 10 min) and eliminate the supernatant.

3. Suspend the S-protein agarose having the target protein bonded thereto in bind/wash buffer (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1 % (v/v) Triton X-100).

4. After centrifuging (500g, 10 min), discard the supernatant to thereby eliminate proteins unspecifically bonded.

5. Suspend the S-protein agarose slurry in 1.5 volumes of elution buffer (bind/wash buffer + 3 M guanidinium thiocyanate, 0.2 M potassium citrate (pH 2), or 3 M $MgCl_2$).

6. Incubate at room temperature for 10 min while occasionally stirring to maintain the suspended state.

7. After centrifuging, harvest the target protein thus eluted.

C. Calmodulin-binding protein (CBP) and calmodulin (CaM)

[0075] In this process, protein is purified by using an interaction between calmodulin-binding protein (CBP) and calmodulin (P. Vaillancourt, Chao-Feng Zheng, D. Q. Hoang, and L. Breister, Affinity Purification of Recombinant Proteins Fused to Calmodulin or to Calmodulin-Binding Peptides. Methods in Enzymology 326: 340-362(2000)). This combination is also

44

usable in the present invention.

[0076] A CBP-fused protein composed of a protein component and CBP bonded thereto is prepared. Then it is purified by adsorbing by a Sepharose 4B-based CaM affinity resin or other commercially available CaM resins. In elution, use is made of EGTA capable of forming a chelate with Ca^{2+}.

[0077] The following protocol shows an example of the process of the preparation of a CBP-fused protein using CBP and CaM. There have been known a number of variations of this process and an appropriate one may be selected therefrom.

1. Add 20 ml of overnight culture of transformed cells (pCA series constructed on the basis of pET-11 as a vector) overnight to 1 l of LB medium containing 50 µg/ml of ampicillin or carbenicillin. Incubate to $OD_{600}=0.6 \sim 10$. After adding IPTG to give a final concentration of 1 mM, continue the incubation for additional 3 to 5 hr under shaking.

2. Harvest the cells by centrifuging. Suspend the cell pellet in buffer A (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM 2-mercapto-ethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$) containing 0.2 mg/ml of lysozyme. Disrupt the cells by sonication.

3. Centrifuge the cell lysate (25,000 g, 15 min) and collect the supernatant.

4. Equilibrate 10 ml of a CaM-Sepharose resin with buffer A.

45

5. Mix the CaM-Sepharose resin with the cell lysate and gently stir for 1 hr. Centrifuge at a low speed and eliminate the slurry to thereby eliminate unspecifically bonded matters.

6. Wash with 40 ml of buffer A. Re-suspend in 20 to 30 ml of buffer A and then pack into a column. Wash successively with 5 column bed volumes of buffer A and buffer B (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM 2-mercapto-ethanol, 1 mM magnesium acetate, 1 mM imidazole, 0.1 mM $CaCl_2$) until A_{280} of the UV detector attains the base line.

7. Elute the target protein with buffer B containing 2mM of EGTA.

D. HSA and ABP

[0078] There has been reported a process for purifying protein by using the binding of human serum albumin (HAS) to serum albumin binding affinity handle (ABP) (T. Graslund, J. Nilsson, A. M. Lindberg, M. Uhlen, and Per-Ake Nygren, Production of a Thermostable DNA Polymerase by Site- Specific Cleavage of A Heat- Eluted Affinity Fusion Protein. Protein Expression and Purification 9: 125-132, 1997). This process comprises expressing a target protein as a fused protein with serum albumin binding affinity handle (ABP), capturing it by an HSA-Sepharose column and eluting with buffer having a low pH value. This combination is also usable in the present invention.

[0079] The following protocol roughly shows this process for

protein purification.

1. Add 5 ml of overnight culture of transformed cells (host: *E. coli*, vector: pET-21a (Novagen)) to 500 ml of TSB + YE medium (30 g/l tryptic soy broth, 5 g/l yeast extract, 100 mg/l ampicillin, 34 mg/l chloramphenicol) followed by incubation under shaking to OD_{600}= 0.8 to 1.5.

2. To induce the expression of the fused protein, add 1mM (the final concentration) of isopropyl β-D-thiogalactoside and continue the incubation for additional 3 to 5 hours.

3. Harvest the cells by centrifuging. Suspend the cell pellet in TST (50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 0.05 % Tween 20, 1 mM EDTA) and disrupt the cells by sonication.

4. Centrifuge the disrupted cell suspension (20,000 g,30min). Filter the supernatant thus obtained through a 1.2 μm-hydrophilic filter.

5. Apply the cell extract to an HSA-Sepharose column.

6. Elute the target protein with a 0.5 M Hac solution (pH 2.8).

(5-4) Process with the use of binding of protein to specific low-molecular weight compound such as amino acid, DNA, dye, vitamin or lectin

A. Glutathione S-transferase (GST) and glutathione

[0080] A process for purifying protein by using an interaction between GST and glutathione, which is called the GST pull down method, has been commonly performed. (see, for example, ,

47

Tanpakushitsu Jikken Noto (Protein Experiment Note) (I), on and after p.162, Chap. 5: 1. GST-Yugo Tanpakusthitu no Hatugen to Seisei (Expression and Purification of GST-fused Protein), Suetake, D. B. Smith, Generating Fusions to Glutathione S-Transferase for Protein Studies. Methods in Enzymology 326:254-270, (2000)). This combination is also usable in the present invention.

[0081] This process comprises preparing a fused protein composed of a target protein with GST and adsorbing by glutathione-agarose employed as an adsorbent. As an eluent, use is made of reduced glutathione. In case of preparing the GST-fused protein by a genetic engineering technique, it is known to use, for example, *E. coli, Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* as the host.

[0082] The following protocol roughly shows an example of the process of the production of GST-fused protein using GST and glutathione. There have been known a number of variations of this process and an appropriate one may be selected therefrom.

1. Dilute 100 ml of overnight culture of transformed cells in 1 l of L medium containing 100 μg/ml of ampicillin followed by incubation.

2. Harvest the cells by centrifuging (5000 g). Suspend the cell pellet in 20 ml of ice-cold PBS containing a reducing agent such as 1 to 5 mM of dithiothreitol (DTT) or 0.1% of 2-mercaptoethanol.

3. Gently sonicate the suspended cells on ice so as that the suspension does not forth. Control the sonication to such a level that the solution turns into dull gray within about 5 min.

4. Add Triton X-100 to give a final concentration of 1% and centrifuge (10,000 g, 4°C, 5 min). Apply the supernatant into a 50 ml tube. Add 1 ml of pre-swollen 50% glutathione-agarose beads and, inverting occasionally, incubate at 4°C for 30 min.

5. Collect the beads by centrifuging (500 g, 30 min) and wash with 50 ml aliquots of ice-cold PBS thrice.

6. Elute the fused protein by gently stirring the beads with an equal volume of freshly made 50 mM Tris-HCl (pH 8) at room temperature for 5 min.

7. Eliminate the supernatant by centrifuging (500 g, 30 sec), add glycerol to give a final concentration of 10% and store in aliquots at -80°C.

B. Protein and dye-ligand

[0083] It is reported that a native protein originating in *Zymononas mobilis* shows an affinity for specific dyes such as C.I. 17908, Reactive Red 8 and C.I. Reactive Blue 187 and this protein can be purified by using this characteristic (R. K. Scopes and K. Griffiths-Smith, Use of Differential and Dye-Ligand Chromatography with Affinity Elution for Enzyme Purification: 6-Phosphogluconate Dehydratase from Zymononas

49

mobilis. Analytical Biochemistry 136: 530-534, 1984).   Namely,
a target protein can be purified by labeling the target protein
with all or a part of this protein, adsorbing by a Sepharose
affinity column using the above dye as a ligand and then eluting.
[0084]   The following protocol shows the process for purifying
the Z. mobilis-origin protein.

1. Starting from a liquid culture of Z. mobilis, prepare
a cell extract with the use of extraction buffer (20 mM K-Mes
(pH 6.5), 30 mM NaCl, 5 mM $MnCl_2$, 0.5 mM ammonium ferrous sulfate,
10 mM β-mercaptoethanol).

2. Apply to the cell extract successively to a Scarlet
MX-G (C.I. 17908, Reactive Red 8)-Sepharose CL-4B column
(Pharamacia) and a Blue HE-G (C.I. Reactive Blue 187)- Sepharose
CL-4B column.

3. Wash the columns with 100 ml of the extraction buffer.

4. Wash not the Scarlet MX-G column but exclusively the
Blue-HE-G column with the extraction buffer containing 20 mM
of Na_2So_4 .

5. Elute the target protein originating in Z. mobilis with
20 mM DL-α-glycerophosphate.

C. Process with the use of binding of biotin to avidin

[0085]   A process for purifying a protein by using the specific
binding of biotin to avidin has been known for a long time (see,
for example, J. D. Alche, and H. Dickinson, Affinity
Chromatographic Purification of Antibodies to a Biotinylated

50

Fusion Protein Expression in *Escherichia coli*. Protein Expression and Purification 12:138-143, 1998). This process comprises preparing a fused protein composed of biotin and a target protein by using a sequence (122 amino acids) which is biotinylated in a host (for example, *E.coli*), capturing this fused protein by a column having avidin fixed thereto (for example, SoftLink soft release avidin resin (Promega)) and then competitively eluting with biotin. This combination is also usable in the present invention.

[0086] The following protocol roughly shows an example of the process with the use of binding of biotin to avidin.

1. To induce gene expression, add 1 mM of IPTG (the final concentration) to overnight culture of transformed cells (host: *E. coli*, vector: PinPoint Xa-2 (Promega)) and incubate for additional 5 hr. In case where the thus expressed protein is present within the cells, treat as follows.

2. Lyse the cells by adding 10 ml per gram of the cell pellet of buffer (50 mM Trin-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 0.1 mM PMSF, 1 mg/ml lysozyme).

3. Collect the precipitate containing the target protein by centrifuging (18,000g, 15 min) and suspend in buffer (50 mM Tris-HCl (pH 8), 10 mM EDTA, 50 mM NaCl, 0.5 % Triron X-100, 0.1 mM PMSF) followed by washing twice.

4. After centrifuging (18,000g, 15 min), suspend the pellet thus obtained in solubilization buffer (50 mM Tris-HCl

51

(pH 8), 10 mM EDTA, 50 mM NaCl, 0.5 % Triton X-100, 0.1 mM PMSF, 6 M guanidine-HCl).

5. Apply the cell extract to 3 ml of a SoftLink soft release avidin resin (Promega) column pre-equilibrated with 30 ml of the solubilization buffer. Wash with 60 ml of the solubilization buffer.

6. Elute the biotinylated target protein with solubilization eluent containing 5 mM of biotin.

(5-5) Process with the use of binding of protein to saccharide

[0087] In this process, a protein is purified by using an interaction between a saccharide-binding protein with a saccharide. For example, it is known to use maltose-binding protein (MBP) together with amylose (D. Sachdev and J. M. Chirgwin, Fusions to Maltose-Binding Protein: Control of Folding and Solubility in Protein Purification. Methods in Enzymology 326: 312-321(2000).) Furthermore, it is expected that use can be made of the interaction between β-galactose-binding proteins such as galectin and β-galactose. These combinations are also usable in the present invention.

A. Maltose-binding protein and amylose

[0088] In the process wherein a fused protein of maltose-binding protein is adsorbed by a resin having amylose fixed thereto, maltose is used as an eluent. The following protocol shows an example of the production process of a fused protein of maltose-binding protein. There have been known a

number of variations of this process and an appropriate one may be selected therefrom.

1. Add 2 ml of overnight culture of transformed cells (*E. coli*/vector pMAL-c2 (New England Biolabs)) to 225 ml of LBD medium containing 100 µg/ml of ampicillin (LB medium containing 0.2% of glucose) and incubate under shaking to OD_{600}=0.5 at 37°C.

2. To induce gene expression, add 0.3 mM of isopropyl-β-thiogalactopyranoside (IPTG) and continue the incubation for additional 2 to 3 hr at 30°C.

3. Harvest the cells by centrifuging (6,800g, 5 min). Suspend the cell pellet in 10 ml of column buffer (20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA, 0.02% Tween 80) and frozen at -20°C overnight.

4. Thaw the frozen cell suspension in ice-water and dilute in 10 ml of the column buffer. Disrupt the cells by sonication (intensity: 75% of the maximum level).

5. After centrifuging (20,000g, 4°C, 15 min), dilute the supernatant with 10 ml of the column buffer (crude extract).

6. Apply the crude extract to 10 ml of an amylose resin column.

7. Wash the column several times with 20 to 30 ml aliquots of the column buffer. Then elute the target protein with the column buffer containing 10 mM of maltose.

B. Chitin and chitin-binding domain (CBD)

[0089] In this process, use is made of the binding of chitin

to chitin-binding domain (CBD) in purifying a protein. Namely, a fused protein composed of chitin-binding protein (CBD) bonded to a target protein via intein (inducible self-cleavage activity of engineered protein splicing elements) is expressed and then adsorbed by a chitin affinity column (New England Biolabs). In elution, the bond between intein and the target protein is cleaved with a reducing agent such as DTT, β-mercaptoethanol orcystein (see, for example, Chung-Mo Park, Jae-Yoon Shim, Song-Sook Yang, Jeong-GuKang, Jeong-Il Kim, Z. Luka, and Pill-Soon Song, Chromophore — Apoprotein Interactions in Synechocystis sp. PCC6803 Phytochrome Cph1. Biochemistry 39: 6349-6356, 2000). In case of applying this process to the protein components of the present invention, the protein components are labeled with an adhesive substance other than CBD-intein too and this adhesive label is used in the separation of the protein components from the target protein formed in the *in vitro* synthesis system.

[0090] The following protocol roughly shows an example of this process for purifying a protein. There have been known a number of variations thereof and an appropriate one may be selected therefrom.

1. Add 3 ml of overnight culture of transformed cells (host: *E. coli*, vector: pTYB2 (New England Biolabs)) to 250 ml of RB medium (0.5 % yeast extract, 1% tryptone, 0.5 % NaCl, 0.2 % glucose (pH 7.5) and incubate to $OD_{600}=0.6$ at 30°C.

2. To induce the expression of the fused protein, add 1 mM of IPTG (the final concentration) and continue the incubation for additional 14 to 16 hr at 20°C.

3. Harvest the cells by centrifuging (5,000g, 5 min). Suspend the cell pellet in lysis buffer (Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 % Triton X-100, 1 mM EDTA) under ice-cooling. Disrupt the cells by sonication.

4. Collect the supernatant by centrifuging (100,000g, 30 min) and filter through a 0.2 mm filter membrane.

5. Add 20 μl of 2 mM DMSO to 1.5 ml of the cell extract. Incubate on ice for 1 hr and then apply to a chitin affinity column.

6. Wash the column with buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100). Incubate in buffer containing 1 mM of DTT (the final concentration) at 4°C overnight to induce the self-cleavage of the intein. Then collect the target protein thus released.

(5-6) Process with the use of binding of protein or peptide
       fragment to ion exchange resin

A. Poly Arg and ion exchange resin

[0091] In this process, protein is purified by taking advantage of the phenomenon that a Poly Arg-tagged target protein, which is charged positively, is adsorbed by a cation exchange resin (for example, an SP-TSK HPLC column). Elution is carried out by controlling the ion strength (J. C. Smith, R. B. Derbyshire,

55

E. Cook, L. Dunthorne, J. Viney, S. J. Brewer, H. M. Sassenfeld, and L. D. Bell, Chemical Synthesis and Cloning of a Poly (Arginine) — Coding Gene Fragment Designed to Aid Polypeptide Purification. Gene 32: 321-327, 1984).

[0092] The following protocol roughly shows an example of the above-described process. There have been known a number of variations thereof and an appropriate one may be selected therefrom.

1. Add 6 ml of overnight culture of transformed cells (host: *E. coli*, vector: pWT221) to 300 ml of M9 medium containing 100 μg/ml of ampicillin and incubate to A_{600}=0.4 under shaking at 37°C.

2. Add an IAA solution (20 mg/ml in ethanol) to give a final concentration of 20 μg/ml.

3. Add 0.1% of Polymin P (the final concentration) and harvest the cells by centrifuging.

4. Lyse the cell pellet in buffer (40 mM Tris-acetate (pH 5.5), 5 M urea) and dialyze against the same buffer.

5. Apply 0.1 ml of the cell extract to an SP-TSK HPLC column and wash the column by the same buffer.

6. Elute the target protein into buffer (40 mM PIPES (pH 6.0), 5 M urea) under an NaCl concentration gradient (100 mM to 350 mM).

(5-7) Process with the use of magnetic beads

[0093] There have been marketed magnetic beads (Dynabeads™;

DYNAL, Norway) having a uniform particle size which consist of a polymer core having magnetizable substances (for example, γFe_2O_3 and Fe_3O_4) uniformly dispersed therein and a hydrophilic polymer coating. By bonding various antibodies to the surface, these beads can be bonded to cells and proteins. When brought close to a powerful magnet (MPC), magnetic beads are magnetized and thus attracted magnetically. When the magnet is removed, the beads are demagnetized and dispersed again. Owing to these characteristics, magnetic beads have been used in, for example, purifying cells and proteins. For example, it is reported that peripheral blood B lymphocytes were isolated by using magnetic polystyrene beads (DYNAL) coated with CD19 antibody (Kanegasaki, S. et al, J.Biochem. 117:758-765(1995)). In the present invention, it is also possible to label the protein components constituting the reaction system with magnetic beads and thus magnetically eliminate from the reaction system. That is to say, such a combination of magnetic beads with a magnet also falls within the category of the substances mutually interacting according to the present invention.

Brief Description of the Drawings

[Fig. 1]

Fig. 1 shows *E. coli* ribosome fractions under a sucrose density gradient.

57

[Fig. 2]

Fig. 2 shows 12% SDS-PAGE patterns of His-tagged initiation factors, elongation factors and termination factors (stained with coomassie brilliant blue).

[Fig. 3]

Fig. 3A shows the relative DHFR activities of His-tagged initiation factors, and Fig. 3B shows the optimum concentrations of His-tagged initiation factors expressed in the relative DHFR activities.

[Fig. 4]

Fig. 4 shows the activities of His-tagged termination factors expressed in the yields of fMFL.

[Fig. 5]

Fig. 5A shows a diagram of UV absorption at 570nm of elute including His-tagged SerRS. Fig. 5B shows a chromatogram of His-tagged SerRS.

[Fig. 6]

Fig. 6 shows 12% SDS-PAGE patterns of His-tagged ARSs and MTF.

[Fig. 7]

Fig. 7A shows the optimum concentrations of His-tagged termination factors in the *in vitro* poly(Phe) synthesis system according to the present invention expressed in the Phe incorporation levels. Fig. 7B shows the progress of poly(Phe) synthesis reactions (expressed in the Phe incorporation levels)

in the *in vitro* synthesis system according to the present invention and in the S100 extract system.

[Fig. 8]

Fig. 8A shows 12% SDS-PAGE patterns of [^{35}S]Met-containing DHFR products, which were synthesized respectively by using the *in vitro* synthesis system according to the present invention and the S30 system. Fig. 8B shows the DHFR activities of these products.

[Fig. 9]

Fig. 9 shows the time courses of the DHFR synthesis reactions in the *in vitro* synthesis system of the present invention and in the S30 system.

[Fig. 10]

Fig. 10 shows the consumption of the energy source in the *in vitro* synthesis system of the present invention (right) and in the S30 system (left) with the passage of time.

[Fig. 11]

Fig. 11 shows the formation of DHFR carrying valine residue incorporated into the 37-position as a model of the incorporation of an unnatural amino acid in the *in vitro* synthesis system of the present invention.

[Fig. 12]

Fig. 12A shows a diagram of UV absorption at 570nm of elute including His-tagged T7RNA polymerase. Fig. 12B shows a chromatogram of His-tagged T7RNA polymerase.

[Fig. 13]

Fig. 13 shows SDS-PAGE patterns of various proteins synthesized by using the *in vitro* synthesis system of the present invention.

[Fig. 14]

Fig. 14 shows the purity of DHFR, which is the translation product in the *in vitro* synthesis system of the present invention, after passing through ultrafiltration membrane with cut-off of 100kDa and a nickel column. In this figure, the arrow shows the position of DHFR.

Detailed Description of the Preferred Embodiments

[0094]   Now, the present invention will be illustrated in greater detail by reference to the following Examples. However, it is understood that the invention is not construed as being restricted to these examples.

[Example 1]

Preparations of E.coli ribosome and extraction of S100

[0095]   300g of *E.coli* A19 cells (harvested at mid-log phase) were ground with alumina. The ground cells were suspended in buffer A (10mM Hepes-KOH (pH7.6), 10mM MgCl$_2$, 50mM KCl, 1mM DTT) and alumina and cell debris were eliminated by centrifugation at 30,000g for 1h at 4℃. DNase (deoxyribonuclease) was added

60

to the resultant supernatant fraction to give a final concentration of 1 µg/ml followed by centrifugation at 100,000g for 4h at 4℃. The supernatant fraction thus obtained was referred to as S100. The pellet was resuspended in the buffer A and the resultant suspension was referred to as a crude ribosome extract. From this crude ribosome extract, a tight-coupled ribosome fraction was obtained under a sucrose density gradient of 6 to 36%. This tight-coupled ribosome fraction was centrifuged at 100,000 g and the pellet was suspended in ribosome buffer (20mM Hepes-KOH (pH7.6), 6mM MgOAc, 30mM NH₄Cl 7mM β-mercaptoethanol) to thereby prepare tight-coupled ribosome. Fig. 1 shows the ribosome fractions under the sucrose density gradient.

[Example 2]

Construction of plasmids for overexpressing initiation factors, elongation factors and termination factors

[0096] Using *E. coli* A19 genome as a template, a gene sequence encoding EF-Tu gene was amplified by PCR to give a DNA fragment having an EcoRI-recognition sequence at the 5′ terminus and a BglII-recognition sequence at the 3′ terminus. The DNA fragment thus obtained was inserted into a plasmid pQE60 (QIAGEN) which had been cleaved with EcoRI and BglII. Thus, a vector for overexpressing EF-Tu fused with His tag at the C terminus was constructed. Next, *E. coli* BL21/pREP4 was

transformed by the vector obtained above. Vectors for overexpressing other elongation factors, initiation factors and termination factors were constructed in the same manner. Table 1 summarizes the vectors and restriction enzymes employed and the His tag sites.

[Example 3]

Construction of plasmids for overexpressing aminoacyl-tRNA synthetase (ARS) and methionine-tRNA formylase (MTF)

[0097] Using *E. coli* A19 genome as a template, a gene sequence encoding alanyl-tRNA synthetase gene was amplified by PCR to give a DNA fragment having an SphI-recognition sequence at the 5' terminus and a HindIII-recognition sequence at the 3' terminus. The DNA fragment thus obtained was inserted into a plasmid pQE30 (QIAGEN) which had been cleaved with SphI and HindIII. Thus, a vector for overexpressing alanyl-tRNA synthetase fused with His tag at the N terminus was constructed. Next, *E. coli* BL21/pREP4 was transformed by the vector obtained above. Vectors for overexpressing other ARS and MTF were constructed in the same manner. Table 1 summarizes the vectors and restriction enzymes employed and the His tag sites, wherein the plasmids obtained were transformed into E.coli BL21/pREP4(pQE series) or BL21/DE3(pET series) strain.

Table 1

62

Enzymes or factors	vector	N-terminal R.E.	C-terminal R.E.	site of His-tag
AlaRS	pQE30	*Sph* I	*Hind* Ⅲ	N
ArgRS	pET16b	*Nde* I	*Bam* H I	N
AsnRS	pQE30	*Bam* H I	*Hind* Ⅲ	N
AspRS	pET21a	*Nde* I	*Xho* I	C
CysRS	pET21a	*Nde* I	*Xho* I	C
GlnRS	pET21a	*Nde* I	*Xho* I	C
GluRS	pET21a	*Nde* I	*Xho* I	C
GlyRS	pET21a	*Nde* I	*Xho* I	C
HisRS	pET21a	*Nde* I	*Xho* I	C
IleRS	pET21a	*Nde* I	*Hind* Ⅲ	N
LeuRS	pET21a	*Xba* I	*Xho* I	C
LysRS	pET21a	*Nde* I	*Xho* I	C
MetRS	pET21a	*Xba* I	*Xho* I	C
PheRS	pQE30	*Sph* I	*Hind* Ⅲ	N
ProRS	pET21a	*Nde* I	*Xho* I	C
SerRS	pET21a	*Xba* I	*Xho* I	C
ThrRS	pQE30	*Bam* H I	*Hind* Ⅲ	N
TrpRS	pET21a	*Nde* I	*Xho* I	C
TyrRS	pET21a	*Nde* I	*Xho* I	C
ValRS	pET21a	*Xba* I	*Not* I	C
MTF	pET21a	*Nde* I	*Xho* I	C
IF1	pQE30	*Bam* H I	*Hind* Ⅲ	N
IF2	pQE30	*Bam* H I	*Hind* Ⅲ	N
IF3	pQE30	*Bam* H I	*Hind* Ⅲ	N
EF-G	pQE60	*Mun* I	*Bgl* Ⅱ	C
EF-Tu	pQE60	*Eco* R I	*Bgl* Ⅱ	C
EF-Ts	pQE60	*Nco* I	*Bam* H I	C
RF1	pQE60	*Bam* H I	*Hind* Ⅲ	C

Note: RE means a restriction enzyme.

[Example 4]

Construction of plasmid for overexpressing T7RNA polymerase

[0098] Using T7 phage genome as a template, a gene sequence encoding T7RNA polymerase gene was amplified by PCR to give a DNA fragment having a BamHI-recognition sequence at the 5′ terminus and a PstI-recognition sequence at the 3′ terminus. The DNA fragment thus obtained was inserted into a plasmid pQE30

(QIAGEN) which had been cleaved with BamHI and PstI. Thus, a vector for overexpressing T7RNA polymerase fused with His tag at the N terminus was constructed. Next, *E. coli* BL21/pREP4 was transformed by the vector obtained above.

[Example 5]

Construction of plasmids for overexpressing nucleoside diphosphate kinase (NDK) and other enzymes

[0099]   Using *E. coli* A19 genome as a template, a gene sequence encoding NDK gene was amplified by PCR to give a DNA fragment having a BamHI-recognition sequence at the 5' terminus and an HindIII-recognition sequence at the 3' terminus. The DNA fragment thus obtained was inserted into a plasmid pQE30 (QIAGEN) which had been cleaved with BamHI and HindIII. Thus, a vector for overexpressing NDK fused with His tag at the N terminus was constructed. Next, *E. coli* BL21/pREP4 was transformed by the vector obtained above. Plasmids for other enzymes exemplified in (4-1) and (4-2) at page 31 can be constructed in the same manner, if desired.

[Example 6]

Overexpression and purification of initiation factors, elongation factors and termination factors

[0100]   To overexpress His-tagged EF-Tu (EF-Tu*), the transformant BL21/pREP4 cells obtained in Example 2 were grown

64

in 6 l of LB medium until the optical density (OD_{660}) attained

0.7.   To the culture, IPTG (isopropyl-1-thio-β-D-galactoside)

was added to give a final concentration of 0.1 mM and incubation

was continued for additional 4 hr at 37°C.   The cells were

harvested by centrifugation, resuspended in suspension buffer

(50mM Hepes-KOH (pH7.6), 1M NH_4Cl, 10mM $MgCl_2$, 0.3mg/ml lysozyme,

0.1% Triton X-100, 0.2mM PMSF (phenylmethanesulfonyl fluoride),

6mM β-mercaptoethanol) and disrupted by sonication. Cell debris

were eliminated by centrifugation at 100,000g for 1 hr at 4°C.

The obtained supernatant was applied to an Ni^{2+} pre-charged 10

ml Hi-Trap chelating column (Pharmacia) and washed with 100 ml

of HT buffer (50mM Hepes-KOH (PH7.6), 1M NH_4Cl, 10mM $MgCl_2$)

containing 10 mM of imidazole.   Then EF-Tu* was eluted from

the column under a linear gradient of imidazole concentration

(10 to 400 mM) contained in the HT buffer.   The EF-Tu* fractions

thus purified were collected and dialyzed against stock buffer

(50mM Hepes-KOH (pH 7.6), 100mM KCl, 10mM $MgCl_2$, 30% glycerol).

The concentration of the purified EF-Tu* was determined based

on a standard curve formed by using the Bio-Rad protein assay

kit using BSA (bovine serum albumin) as a standard.   The

obtained EF-Tu* was quickly frozen in 1 ml aliquots in liquid

nitrogen and then stored at −80°C.   Other His-tagged elongation

factors, initiation factors and termination factors were

purified in the same manner.   Fig. 2 shows 12% SDS-PAGE patterns

of His-tagged factors (stained with coomassie brilliant blue).

[0101]   The activities and optimum concentrations of the His-tagged initiation factors (IF1, IF2 and IF3: "*" means "His-tagged") were measured with the use of a DHFR mRNA *in vitro* translation system (Example 17 provided hereinafter).   Using a system containing all of IF1*, IF2* and IF3* as a positive control, incubation was carried out for 30 min in systems lacking respective initiation factors and then the relative activities of DHFR thus formed were compared (Fig. 3A).   Namely, the activities of the His-tagged initiation factors were determined by referring the activity of the positive control as to 100.   As a result, it was confirmed that the DHFR yield in each of the IF*-lacking systems corresponded to 1/2 or less of the positive control, which indicates that all of IF1*, IF2* and IF3* have the activity.   The optimum concentrations of the His-tagged initiation factors were measured by translating in an *in vitro* system under constant conditions but varying the concentration of each initiation factor and measuring the relative activity of DHFR thus formed (Fig. 3B).   In Fig. 3B, ●, ▲ and ■ respectively show the data of IF1*, IF2* and IF3*.

[Example 7]

Overexpression and purification of His-tagged ARSs and MTF

[0102]   Transformant BL21/DE3 cells for overexpressing His-tagged Ser tRNA synthetase ("*" means "His-tagged") were grown in 2 l of LB medium until the optical density (OD_{660})

attained 0.7. To the culture, IPTG (isopropyl-1-thio- β-D-galactoside) was added to give a final concentration of 0.1 mM and incubation was continued for additional 4 hr at 37℃. The cells were harvested by centrifugation, resuspended in suspension buffer (50mM Hepes-KOH (pH7.6), 1 M NH$_4$Cl, 10 mM MgCl$_2$, 0.3mg/ml lysozyme, 0.1% Triton X-100, 0.2 mM PMSF (phenylmehtanesulfonyl fluoride), 6 mM β-mercaptoethanol) and disrupted by sonication. Cell debris were eliminated by centrifugation at 100,000g for 1 hr at 4℃. The obtained supernatant was applied to an Ni^{2+} pre-charged 10 ml Hi-Trap chelating column (Pharmacia) and washed with 100 ml of HT buffer (50 mM Hepes-KOH (PH7.6), 1 M NH$_4$Cl, 10 mM MgCl$_2$) containing 10 mM of imidazole. Then Ser tRNA synthetase* was eluted from the column under a linear gradient of imidazole concentration (10 to 400 mM) contained in the HT buffer. The Ser tRNA synthetase* fractions thus purified were collected and dialyzed against stock buffer (50 mM Hepes-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl$_2$, 30% glycerol). The concentration of the purified Ser tRNA synthetase* was determined based on a standard curve formed by using the Bio-Rad protein assay kit using BSA (bovine serum albumin) as a standard. The thus obtained Ser tRNA synthetase* was quickly frozen in 1 ml aliquots in liquid nitrogen and then stored at -80℃. Fig. 5 shows a chromatogram of the Ser tRNA synthetase* thus obtained.

[0103] Other ARS* and MTF were overexpessed and purified in

67

the same manner. Fig. 6 shows 12% SDS-PAGE patterns of the His-tagged factors and enzymes (stained with coomassie brilliant blue). Two bands of Gly RS* and Phe RS* observed in Fig. 6 are assignable to the fact that these enzymes have α2 and β2 types. Fig. 6 indicates that these His-tagged factors and enzymes were obtained each at a high purity.

[Example 8]

Overexpression and purification of His-tagged T7RNA polymerase

[0104]   Transformant BL21/pREP4 cells for overexpressing His-tagged T7RNA polymerase ("*" means "His-tagged") were grown in 6 l of LB medium until the optical density (OD_{660}) attained 0.7. To the culture, IPTG (isopropyl-1-thio-β-D-galactoside) was added to give a final concentration of 0.1 mM and incubation was continued for additional 4 hr at 37℃. The cells were harvested by centrifugation, resuspended in suspension buffer (50 mM Hepes-KOH (pH7.6), 1 M NH_4Cl, 10 mM $MgCl_2$, 0.3mg/ml lysozyme, 0.1% Triton X-100, 0.2 mM PMSF (phenylmethanesulfonyl fluoride), 6 mM β-mercaptoethanol) and disrupted by sonication. Cell debris were eliminated by centrifuging the disrupted cell suspension at 100,000g for 1 hr at 4℃.   The obtained supernatant fraction was applied to an Ni^{2+} pre-charged 10 ml Hi-Trap chelating column (Pharmacia) and washed with 100 ml of HT buffer (50 mM Hepes-KOH (PH7.6), 1 M NH_4Cl, 10 mM $MgCl_2$) containing 10 mM of imidazole.   Then T7RNA polymerase* was

68

eluted from the column under a linear gradient of imidazole concentration (10 to 400 mM) contained in the HT buffer. The T7RNA polymerase* fractions thus purified were collected and dialyzed against stock buffer (50 mM Hepes-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl$_2$, 30% glycerol). The concentration of the purified T7RNA polymerase* was determined based on a standard curve formed by using the Bio-Rad protein assay kit using BSA (bovine serum albumin) as a standard. The obtained T7RNA polymerase* was quickly frozen in 1 ml aliquots in liquid nitrogen and then stored at -80℃. Figs. 12A and B show chromatograms of the T7RNA polymerase* thus obtained.

[Example 9]

Overexpression and purification of His-tagged NDK and other enzymes

[0105]   Transformant BL21/pREP4 cells for overexpressing His-tagged NDK ("*" means "His-tagged") were grown in 2 l of LB medium until the optical density (OD$_{660}$) attained 0.7. To the culture, IPTG (isopropyl-1-thio-β-D-galactoside) was added to give a final concentration of 0.1 mM and incubation was continued for additional 4 hr at 37℃.

[0106]   The cells were harvested by centrifugation, resuspended in suspension buffer (50 mM Hepes-KOH (pH7.6), 1 M NH$_4$Cl, 10 mM MgCl$_2$, 0.3mg/ml lysozyme, 0.1% Triton X-100, 0.2 mM   PMSF   (phenylmethanesulfonyl   fluoride),   6   mM

β-mercaptoethanol) and disrupted by sonication. Cell debris were eliminated by centrifugation at 100,000g for 1 hr at 4°C. The obtained supernatant fraction was applied to an Ni^{2+} pre-charged 10 ml Hi-Trap chelating column (Pharmacia) and washed with 100 ml of HT buffer (50 mM Hepes-KOH (PH7.6), 1 M NH_4Cl, 10 mM $MgCl_2$) containing 10 mM of imidazole. Then NDK* was eluted from the column under a linear gradient of imidazole concentration (10 to 400 mM) contained in the HT buffer. The NDK* fractions thus purified were collected and dialyzed against stock buffer (50 mM Hepes-KOH (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 30% glycerol). The concentration of the purified NDK* was determined based on a standard curve formed by using the Bio-Rad protein assay kit using BSA (bovine serum albumin) as a standard. The obtained NDK* was quickly frozen in 1 ml aliquots in liquid nitrogen and then stored at −80°C. Other His-tagged enzymes exemplified in (4-1) and (4-2) at page 31 can be obtained in the same manner, if desired.

[Example 10]

Construction of DHFR gene and preparation of mRNA

[0107] By adding an HindIII sequence and a BamHI sequence respectively to the 5′ and 3′ termini, DHFR (dihydrofolate reductase) gene originating in *E.coli* was amplified by PCR. The gene contained a T7 promoter upstream of a ribosome-binding site with the "epsilon sequence" originating from

70

bacteriophage T7 gene 10 followed by a Shine-Dalgarno (SD) sequence. This DNA fragment was cloned into a plasmid vector pUC18 (Takara Shuzo). After treating with SmaI, this plasmid was used as a template in run-off transcription or *in vitro* transcription coupled translation with the use of T7RNA polymerase. The an *in vitro* transcription reaction was carried out at 42°C for 3 hr. The reaction mixture (1ml) comprised 40mM Hepes-KOH (pH 7.8), 20 mM of $MgCl_2$, 1 mM of spermidine, 5 mM of DTT, 2 mM each of ATP, UTP, CTP, and GTP, 20 µg of the SmaI-treated template plasmid, 50 µg of BSA, 1.78 units of PPiase (pyrophosphatase) and 10 µg of His-tagged T7RNA polymerase thus purified. To cease the reaction, EDTA (ethylenedinitrotetracetic acid) was added to give a final concentration of 50 mM. The mRNA thus obtained was extracted with phenol/chloroform and then precipitated with ethanol. Next, it was purified by using an RNA purification kit (QIAGEN) in accordance with the protocol recommended by the manufacturer.

[Example 11]

Construction of MFL mRNA

[0108] To obtain a template for MFL mRNA, a DNA sequence AUGUUCUUGUAA (translated into fMet-Phe-Leu-Stop; formylmethionine-phenylalanine-leucine-stop codon; hereinafter referred to simply as MFL) was constructed as

follows. An oligonucleotide A: 5′-TAtgttcttgtaac was annealed

with another oligonucleotide B: 5′-TCGAgttacaagaaca to give a

double-stranded DNA containing NdeI and XhoI sequences. Next,

this DNA was cloned into the NdeI and XhoI sites of a plasmid

vector pET29a (Novagen). The resultant plasmid transcribed

as in the above-described case of DHFR gene.

[Example 12]

His-tagged aminoacyl-tRNA synthetase activities

[0109]   Activities   of   His-tagged   ARSs   (aminoacyl-tRNA

synthetases) were measured as follows.   Each reaction mixture

(50 μ l) comprised polymix buffer (see translation experiments)

containing 1 mM of ATP, 2.8A_{260} units of tRNAmix (Boehringer),

50 μ M of each labeled amino acid, and each purified His-tagged

ARS. Reactions were carried out at 37 ℃ and radioactive

aminoacyl-tRNAs were precipitated on 3MM filters at different

incubation times and washed with cold 5% trichloroacetic acid

followed by the measurement of the radioactivity. One unit of

the activity was defined as the amount of the enzyme capable

of synthesizing 1 pmol of aminoacyl-tRNA per minute. Table 2

shows the results.

Table 2

Enzyme	Concentration (μg/μl)	Specific activity (U/μg)	Necessary units (50μl reaction)
AlaRS	13	27	94
ArgRS	10	1300	130
AsnRS	30	ND	ND
AspRS	22	310	130
CysRS	25	500	31
GlnRS	36	330	63
GluRS	26	150	94
GlyRS	30	520	250
HisRS	30	1600	31
IleRS	20	63	130
LeuRS	22	940	190
LysRS	35	580	190
MetRS	27	3000	310
PheRS	23	15	63
ProRS	16	120	63
SerRS	17	1000	94
ThrRS	19	200	63
TrpRS	11	600	31
TyrRS	22	1800	31
ValRS	20	1700	156
MTF	12	230	230

[Example 13]

His-tagged methionyl-tRNA transformylase activities

[0110]   Activities of His-tagged MTFs were measured as follows
("*" means "His-tagged"). Each reaction mixture (50 μ l)
comprised polymix buffer (see translation experiments)
containing 1mM ATP, 2.8 A_{260} units of tRNAmix (Boehringer), 50
μM of [^{3}H] labeled methionine, 0.5 μg of
10-formyl-5,6,7,8,-tetrahydrofolic acid, 3000 units of MetRS,
and MTF*. Reactions were carried out at 37℃ and unformylated
methionyl-tRNAs were deacylated in buffer containing 0.175 M
of $CuSO_4$ and 0.5 M of Tris-HCl (pH7.5) for 8 min at 30℃.

73

Radioactive formyl-methionyl-tRNAs were precipitated on 3MM filters and washed with cold 5% trichloroacetic acid followed by the measurement of the radioactivity. One unit of activity was defined as the amount of enzyme capable of synthesizing 1 pmol of formyl-methionyl-tRNA per minute. Table 2 shows the results (the last column).

[Example 14]

Translation experiments (general procedure)

[0111] Translation mixtures (50 µl) were prepared by slightly modifying polymix buffer employed by Jelenc et al. (1979) and Wagner et al. (1982). The polymix buffer contained 5 mM of magnesium acetate, 5 mM of potassium phosphate (pH7.3), 95 mM of potassium glutamate, 5 mM of ammonium chloride, 0.5 mM of calcium chloride, 1 mM of spermidine, 8 mM of putrescine and 1 mM of DTT. Each reaction mixture contained 1 mM of ATP, 1 mM of GTP, 10 mM of creatine phosphate, 2.8 A_{260} units of tRNAmix, 0.5 µg of 10-formyl-5,6,7,8,-tetrahydrophilic acid, 1 mM of each of amino acid and the factor mix as will be described hereinafter. In case of transcription coupled translation reaction, 1 mM of each NTP and 4 mM of magnesium acetate were further added to the above reaction mixture. The factor and enzyme mix comprised 12 pmol of ribosome, 1 µg of IF1*, 2 µg of IF2*, 0.75 µg of IF3*,1 µg of EF-G*, 2 µg of EF-Tu*, 1 µg of EF-Ts*, 0.5 µg of RF1*, 0.5 µg of RF3*, 0.5 µg of RRF*, 30

74

to 300 units of each ARS* or MTF, 0.2 µg of creatine kinase (CK),

0.15 µg of myokinase (MK) and 0.054 µg of nucleoside diphosphate

kinase* (NDK). In case of transcription coupled translation,

1.78 units of PPiase and 0.5 µg of T7RNA polymerase* were further

added. Among the factors and enzymes, those marked "*" mean

His-tagged ones. Reaction mixtures were incubated at 37℃ for

5 min, then template DNA or RNA was added and reaction was started.

Translation was carried out at 37℃. After the completion of

the reaction, ribosome having high molecular weight was first

eliminated by passing through a ultrafiltration membrane with

a cut-off of 100 kDa. Next, the fraction having passed through

the ultrafiltration membrane was applied to an Ni column and

thus the His-tag fusion proteins were eliminated. The

component passing through the column was the translation

product having a high purity which showed a single band in

SDS-PAGE. The S30 system employed in the following experiments

was purchased from Promega and translation was carried out in

accordance with to the protocol recommended by the

manufacturer.

[Example 15]

Expression of various proteins

[0112] After confirming the activities of the His-tagged

constituents of the reaction system, an *in vitro* protein

synthesis system was constructed as in Example 14 with the use

75

of His-tagged T7RNA polymerase. Using this synthesis system, full-length polypeptides of *E. coli* DHFR, λ-lysozyme, green fluorescent protein (GFP), glutathione transferase (GST) and T7 gene 10 protein were synthesized and the yield of each product was measured. Fig. 13 shows the results. Thus, it has been clarified that the synthesis system according to the present invention contains all of the components required in translation.

[Example 16]

Poly(U)-poly(Phe) synthesis

[0113]   Poly(U)-poly(Phe) was synthesized in an *in vitro* reaction system as follows. Each reaction mixture comprised polymix buffer containing 1 mM of ATP, 1 mM of GTP, 10 mM of creatine phosphate, $2.8A_{260}$ units of tRNAmix, 1 mM of $[^{14}C]$ labeled phenylalanine, and a factor mix. The factor mix contained 12 pmol of ribosome, 1 μg of EF-G*, 2 μg of EF-Tu*, 1 μg of EF-Ts*, 60 units of PheRS*, 0.2 μg of creatine kinase (CK), 0.15 μg of myokinase (MK) and 0.054 μg of nucleotide diphosphate kinase* (NDK*). Among the factors and enzymes, those marked "*" mean His-tagged ones. Reaction mixtures were incubated at 37℃ for 5 min, then 5 μg of poly(U) was added and reaction was started. Poly(Phe) was sampled in 8 μl aliquots with the passage of time and precipitated on 3MM filters with 10% trichloroacetic acid. Aminoacyl-tRNAs were deacylated at

85℃ and washed with 10% trichloroacetic acid followed by the measurement of radioactivity. Thus, the formation of the target product was confirmed.

[0114] In the translation reactions as described above, the optimum concentrations of EF-G*, EF-Tu* and EF-Ts* were determined by examining the poly(Phe) yield in the *in vitro* reaction system under constant conditions but varying the concentration of each elongation factor. Fig. 7(A) shows the results thus obtained. In Fig. 7(A), ●, ▲ and ■ show the data of EF-G*, EF-Tu* and EF-Ts*, respectively.

[0115] Further, the data of the poly(Phe) synthesis in the above-described reaction system according to the present invention were compared with the data in the translation system with the use of the S100 extract. Fig. 7(B) shows the result. Although the reaction stopped after 20 min in the latter system (○), the reaction proceeded even after 40 min in the system of the present invention (●).

[Example 17]

Activities of termination factors and ribosome recycling factor

[0116] Activities of termination factors (RF1*, RF3* and RRF*; "*" manes "His-tagged") were measured according to Pavrov et al. (8) with slight modification. Reaction mixture (50 μl) were prepared based on the polymix buffer used in the translation experiment. Each reaction mixture comprised 1 mM of ATP, 1 mM

of GTP, 2.8A_{260} units of tRNA mix, 1 mM of phenylalanine and leucine, 50 pmol of formylmethionyl-tRNA prepared by using [^{35}S] radioactive methionine and a His-tagged factor and enzyme mix (described hereinafter). The factor and enzyme mix comprised 12 pml of ribosome, 1 µg of IF1*, 2 µg of IF2*, 0,75 µg of IF3*, 1 µg of EF-G*, 2 µg of EF-Tu*, 1 µg of EF-Ts*, 0.5 µg of RF1*, 0.5 µg of RF3*, 0.5 µg of RRF*, 50 units of PheRS* and 300 units of LeuRS*. RF1*, RF3* and RRF* were each removed from this factor and enzyme mix depending on the purpose to give respective reaction mixtures. Each reaction mixture was pre-incubated at 37℃ for 5min, and then 1 µg of MFL mRNA was added to initiate the translation. The reaction mixture was sampled in 5 µl aliquots with the passage of time and each sample was added to the same volume of 1 N HCl to cease the reaction. Further, 200 µl of ethyl acetate was added to elute the tripeptide (fMFL) and the radioactivity was measured with a liquid scintillation counter.

[0117] The activities of the termination factors RF1*, RF3* and RRF* were measured by using an *in vitro* translation system for synthetic mRNA encoding fMet-Phe-Leu-Stop (fMFL). Namely, fMFL mRNA was translated in a system containing RF1*, RF3* and RRF* as termination factors (●), a system containing RF1* and RRF* (·), a system containing RF1* and RF3* (▲), a system containing RF1* alone (■), a system containing RF3* and RRF* (○) and a system free from any termination factor* (RRF* too)

(◆) and the yields were measured. Fig. 4 shows the results. In Fig. 4, the peptide synthesized in the first cycle corresponds to about 1000 cpm on the y-axis. The system containing RF1*, RF3* and RRF* (●) shows a linear increase in the fMFL yield with the passage of time, which indicates that RF1*, RF3* and RRF* have the activities compared with other systems. In the system lacking RF1* (○), no peptide synthesis occurred. In the systems lacking RRF* (▲■◆), no ribosome recycling occurred.

[Example 18]

DHFR synthesis

[0118] DHFR containing [^{35}S] methionine was synthesized by using the *in vitro* translation system according to the present invention and another translation system with the use of the S30 system (Promega). Each product was separated by 12% SDS-PAGE (sodium dodecyl suflate polyacrylamide gel electrophoresis) and detected by a BAS-1000 system (Fuji Film) followed by the measurement of the radioactivity. Fig. 8(A) shows the results of the separation by SDS-PAGE.

[0119] On the other hand, the activity of DHFR was measured as follows. In a reaction mixture containing 50mM potassium phosphate buffer (pH7.0), 50 μM of DHF (dihydrofolic acid) and 60 μM of NADPH (reduced nicotinamide adenine dinucleotide phosphate), DHFR was synthesized at 30°C and the decrease in

79

A_{340} was measured every 1 min. Fig. 8(b) shows the results.

[0120] Fig. 9 shows the reaction processes in the *in vitro* translation system according to the present invention and the S-30 system. In the *in vitro* translation system of the present invention (■), the reaction proceeded even after 120 minutes, while the DHFR yield attained the peak after 20 minutes in the S-30 system (●).

[0121] To examine energy consumption, nucleoside triphosphate was hydrolyzed in the *in vitro* translation system according to the present invention and in the S-30 system. The hydrolysis in each system was monitored in the following manner while comparing the data. Using DHFR templates, translation was carried out in reaction mixtures containing [α-^{32}P] ATP or GTP at 37°C. Each reaction mixture was sampled in 2 μl aliquots with the passage of time and each sample was added to 150 μl of 10% formic acid. Then the mixtures were spotted on a polyethyleneimine TLC plate and the reaction products were developed with the use of 0.75M of potassium phosphate buffer (pH 3.75). After air-drying, the TLC plate was covered with a plastic wrap and autoradiographed. Fig. 10 shows the results wherein the data of the S-30 system are given left while the data of the invention system are given right. In the S-30 system, ATP was decreased with the passage of time, while the amount of ATP was maintained at an almost constant level in the system according to the present invention.

80

[0122] To purify the DHFR synthesized, ribosome was eliminated by using an ultrafiltration membrane with a cut-off of 100 kDa. Next, all of the His-tagged components constituting the reaction system were eliminated by passing the reaction mixture through a nickel column. The reaction mixture before passing through the nickel column and the product obtained after passing through the nickel column were developed by 12% SDS-PAGE and stained with coomassie blue. Fig. 14 shows the results wherein lane 1 stands for the markers, lane 2 stands for the reaction mixture and lane 3 stands for the products (DHFR), indicating that the product was obtained as a single band.

[Example 19]

Valine residue incorporation by valyl suppressor tRNA (model of incorporation of unnatural amino acid)

[0123] In the *in vitro* translation system according to the present invention containing RF2* ("*" means "His-tagged") as a substitute for RF1* as a termination factor, a DHFR template, in which Asn at the 37-position (ATA codon) had been converted into UAG codon, was translated by using a chemically synthesized valyl suppressor tRNA. As a result, a truncated protein terminating at the 37-residue was synthesized in the sample containing RF1* (Fig. 11, lane 2), while a faint band assignable to this truncated protein was observed in the RF1*-free system (containing no RF2) (Fig. 11, lane 3). By incorporating RF2*

thereinto, a protein product was observed at the same position (Fig. 11, lane 4) as normal DHFR (Fig. 11, lane 1). Based on these results, it was confirmed that the valine residue attached to suppressor tRNA had been incorporated into the 37-position of DHFR.

Effects of the Invention

[0124] By labeling protein components constituting a reaction system, individual protein components constituting the reaction system can be surely purified and thus a reaction system contaminated with no unknown component can be established. Moreover, it becomes possible thereby to easily isolate a target protein thus synthesized at a high purity.

[0125] Although it has been pointed out that lipopolysaccharides (LPSs) contained in cells and cell extracts exert various undesirable effects as endotoxins on living body, there is a technical problem that these LPSs can be hardly separated from target peptide products. However, this problem can be solved by using the *in vitro* peptide synthesis system according to the present invention.

[0126] Owing to the establishment of a reaction system free from any unknown components, a reaction can be continued over a long time, for example, 2 hr or longer even in a batch system. Furthermore, it is regarded as theoretically possible to

increase the volume of a reaction mixture.

[0127] Using a flow system, the reaction can be continued for a longer time, which enables the practical production and purification of a protein in an *in vitro* reaction system. That is to say, this process makes it possible to economically supply certain enzymes which have been hesitantly applied to medical treatments because of high cost. As a result, the application range of medical treatments with the administration of enzymes, which are employed as a substitute for at least a part of gene therapy suffering from a technical problem in delivery, can be broadened.

[0128] According to the present invention, a system containing termination factors and a termination factor-free system can be definitely established and thus ribosome displays can be easily and selectively produced. Furthermore, it becomes possible to more accurately incorporate unnatural amino acid residues to desired positions.

[0129] Conventional cell-free protein synthesis systems with the use of prokaryotic cell extract suffer from a problem that the stability of an mRNA is seriously lowered when transcription and translation are not carried out simultaneously. In contrast, a reaction of translating an mRNA can stably proceed in the reaction system of the present invention.

[0130] After the completion of genome analyses, the mainstream of molecular biological studies is now switching over to gene

83

analyses.   Under these circumstances, the reaction system of

the present invention, which quickens gene expression and

identification of protein products and thus facilitates

examination of gene functions, considerably contributes to

advances in scientific technology.

Reference

1)Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D.,

and Henco, K. (1994). 6xHis-Ni-NTA chromatography as a superior

technique in recombinant protein expression/purification.

Methods Mol Biol *31*, 371-87.

2)Hochuli, E., Dobeli, H., and Schacher, A. (1987). New metal

chelate adsorbent selective for proteins and peptides

containing neighbouring histidine residues. J Chromatogr *411*,

177-84.

3)Smith, D. B., and Johnson, K. S. (1988). Single-step

purification of polypeptides expressed in Escherichia coli as

fusions with glutathione S-transferase. Gene *67*, 31-40.